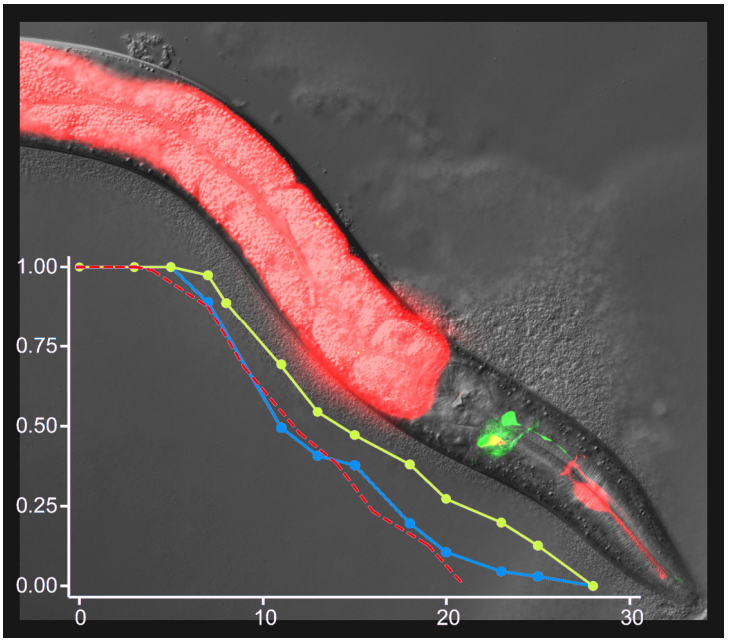
A Regulated Response to Mitochondrial Dysfunction Modulates Longevity and Rates of Living



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Dedicado ao meu avô,
Gregório Zeferino e ao meu
primeiro professor de Biologia,
Joaquim Vicente.

To my grandfather,
Gregório Zeferino and my
first Biology teacher,
Joaquim Vicente.

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Abstract

Aging is a long-standing biological question of tremendous social and cultural importance. Despite this, only in the last 15 years has biology started to make significant progress in understanding the underlying mechanisms that regulate aging. This progress stemmed mainly from the use of model organisms, which allowed the discovery of several genes directly modulating longevity. Interestingly, several of these longevity genes are necessary for normal mitochondrial function, and disruption of their activity delays the aging process. This is somewhat paradoxical, considering the importance of cellular respiration for energy production and viability of eukaryotic organisms. One possible rationalization for this is that by decreasing cellular respiration, reactive oxygen species (ROS) generation is also reduced, and in that way, cellular decay and aging are delayed. The mechanism, however, may not be this simple, since it is unclear whether impairing mitochondrial respiration reduces ROS, and also, since not all long-lived mitochondrial mutants tested are resistant to ROS. Besides the long-life phenotype, these mitochondrial mutants also show a significant “slowing down” of rates of living (defecation, moving, eating and development to adulthood). Initial analysis of these phenotypes led to the suggestion that the increased longevity was not a regulated event, and instead was a consequence of a passive “slowing down” of the animals due to less energy availability. However, mitochondrial defects can only increase longevity if present during development, which in itself suggests the existence of a regulatory event. In order to understand the mechanisms by which mitochondrial impairment could be leading to increased longevity, we used the nematode *C. elegans* and performed gene expression analysis using microarrays, followed by functional analysis of candidate genes using RNAi. These studies suggested that mitochondrial mutations are affecting

longevity by up-regulating cellular defenses against toxic molecules and by activating alternative metabolic pathways. Interestingly, this is highly reminiscent of a transcriptional response observed in long-lived yeast with impaired mitochondria. This, in turn, strongly suggests evolutionary conservation of this pathway. When we looked for genes that were necessary for the increased longevity of mitochondrial mutants we identified the first regulator of this response, *fstr-1/2*. Importantly, *fstr-1/2* is necessary for the “slowness” to occur in animals with defective mitochondria, which demonstrates that the response to mitochondrial dysfunction is actively regulated. This work also demonstrates that there are significant differences in how distinct mitochondrial mutants regulate their responses, although the overall nature of their responses is similar.

Sumário

O envelhecimento é uma questão biológica antiga e de grande importância sócio-cultural. Apesar disto, os progressos mais significativos a nível mecanístico no campo da biologia do envelhecimento só se fizeram sentir nos últimos 15 anos. Estes progressos partiram, principalmente, da utilização de organismos modelo que permitiram a identificação de genes directamente envolvidos na regulação da longevidade. Muitos destes genes são necessários para o funcionamento normal das mitocôndrias e a inibição da sua função leva a um retardamento do processo de envelhecimento. Esta observação é algo inesperada, pois a respiração celular que se desenvolve nas mitocôndrias é extremamente importante para a produção energética e para a viabilidade das células eucarióticas. Uma razão possível para esta observação é que com a redução da respiração celular, também ocorre uma redução na produção de radicais livres (ROS) e, desse modo, a degeneração celular e o envelhecimento são adiados. No entanto, o mecanismo real deverá ser mais complexo pois, por um lado, a inibição da respiração celular nem sempre reduz os níveis de radicais livres, e por outro, nem todos os mutantes mitocondriais apresentam resistência a radicais livres. Para além do aumento no tempo de vida, estes mutantes mitocondriais também apresentam um "abrandamento" considerável na suas taxas de vida (defecação, movimento, alimentação, e desenvolvimento até adultos). Uma análise inicial destes fenótipos poderá sugerir que o aumento de vida observado nestes mutantes mitocondriais não é regulado biologicamente e é apenas uma consequência dum "abrandamento" passivo e generalizado dos animais devido a uma redução de produção energética. No entanto, esta explicação poderá ser demasiado simples, pois deficiências mitocondriais só afectam a longevidade se presentes durante o desenvolvimento pré-

embrionário, o que por si só, sugere a presença de um mecanismo regulador. Para compreender melhor os mecanismos responsáveis pelo aumento de longevidade em resposta a deficiências mitocondriais, nós focamo-nos no organismo modelo *C. elegans*. Analisamos os perfis de expressão génica destes mutantes usando "microarrays", e seguidamente fizemos uma análise funcional dos genes com expressão alterada usando RNA interferência (RNAi). Estes estudos sugerem que as perturbações mitocondriais estão a afectar a longevidade através dum aumento da expressão de genes com funções protectoras contra moléculas tóxicas e, também, através da utilização de vias metabólicas alternativas. É importante notar que este fenómeno é muito semelhante à resposta transcricional observada em leveduras com deficiências mitocondriais que também têm vida prolongada. Esta observação é um forte indício de que este mecanismo de regulação de longevidade em resposta a deficiências mitocondriais é evolutivamente conservado. Quando procurámos genes necessários para o aumento do tempo de vida observado em mutantes mitocondriais, encontrámos o *fstr-1/2*, o primeiro regulador deste mecanismo descrito até agora. É de notar que a actividade deste gene é necessária para o "abrandamento" observado nestes animais, o que por si só, demonstra que esta resposta à inibição mitocondrial é regulada e não apenas uma consequência passiva de falta de energia. Este trabalho também evidencia diferenças ao nível da regulação da resposta transcricional entre os diferentes tipos de mutantes mitocondriais em *C. elegans*. No entanto, apesar dos mecanismos de regulação serem diferentes, as respostas transcricionais dos diferentes mutantes são semelhantes. Isto sugere que estes mutantes mitocondriais activam respostas semelhantes que regulam a longevidade através de mecanismos diferentes.

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Chapter I

Introduction

"To get back my youth I would do anything in the world, except take exercise, get up early, or be respectable." – Oscar Wilde

Aging is a widespread process with deep-rooted implications in our lives and societies. Humanity has struggled to understand this mystery and its implications for millennia, and even presently, many questions remain. It has captured the imagination of many philosophers and artists. Great literary works were created either embracing the inevitability of aging or fantasizing about eternal youth. The idea of prolonged life is a common mythological theme in many cultures, and an intrinsic part of our collective imagination. More so, the search for long life is not a recent trend, in fact, Juan Ponce de Leon, an influential Spanish explorer, in 1513 found himself dissatisfied with his riches and set out on a quest for the fountain of youth, discovering Florida in the process (Oviedo 1535; Gómara 1551). Already many years before this "conquistador's" adventure, in the 1300s, alchemy attempted to, besides the noble goal of turning household items into gold, create a liquid "panacea" that would heal all illnesses and allow eternal life (Burland 1989).

However, besides the more philosophical, historical and imaginative aspects of aging, there are present day socio-economical implications that need to be understood and acted upon. Many countries in the developed world, mainly Western Europe and Asia, due to improved medical and sanitary conditions and decreased birth rates, have increasingly aged populations. This inflates the social burden due to higher health care costs (Saltman, Dubois et al. 2006). In addition, there is a significant decrease in

economic growth since the main consumer base consists of younger people (Krulwich 2006). Also, as the fraction of elderly individuals increases, it is important to understand the underlying physiological changes of aging and age-related disease, developing appropriate medical procedures in the process.

More relevantly to this work, the aging process represents a major unanswered biological question of tremendous scientific interest and potential. A complex task that requires an interdisciplinary approach, its study generates a better understanding of both fundamental biological processes and age-related disease. It is a compelling question that fuels the imagination of scientists and non-scientists alike.

1.1 - Aging in Biology – *"What is aging?"*

There is no simple definition since there are several phenotypes associated with aging, some of which are shared between a wide range of species, some of which are unique. However, aging can be defined broadly as the age-associated decline of physiological and neurological processes leading to a decrease in overall function of an organism.

It is important to note that bacteria and certain mammalian cells in culture have the ability to divide almost indefinitely, so, clearly, the potential to keep cellular stability through time exists. Yet, despite of this, most organisms lose function, age and eventually die. So the question remains, why do organisms age?

1.2 - Theories of Aging – *"Why do we age?"*

Evolutionary biologists have considered the aging problem extensively and have developed several compelling theories. Weissman initially proposed (Weismann, 1891) that aging is a genetically programmed means of controlling population size and avoiding overcrowding and depletion of

resources. Along these lines, a commonly heard argument for aging as an evolutionarily selected process is that it increases generation turnover, thus facilitating adaptation to changing environments. This theory is no longer believed to be accurate, mostly due to three evolutionary arguments: Firstly, in the wild, animals tend to die early from extrinsic causes (predation, climate, disease), thus making the effects of aging as a population control mechanism negligible (Medawar, 1952). Secondly, since the majority of the animals die young, natural selection cannot act on phenotypes that occur later in life. Thirdly, because senescence is mainly post-reproductive, mutations that affect this life period are less likely to be selected for, since they should have minimal effects on fitness. Hence, it is hard to imagine how a mechanism with no function other than to regulate longevity may have been selected for. This does not mean, however, that genes cannot affect longevity, but that aging *per se* was not evolutionarily selected for.

Along these lines, we have a more widely accepted theory of aging, the mutation accumulation theory of aging. This theory states that because natural selection cannot act later in life, populations will tend to randomly accumulate mutations that will have deleterious effects in older organisms. The mutations that act the latest are exposed to lower selective pressures, which allows an accumulation of progressively worse phenotypes as animals age (Kirkwood 2002). This theory predicts that over the span of many generations there will be a severe decrease in overall function in older organisms, in other words, aging.

An interesting follow-up to the mutation accumulation theory is the antagonistic pleiotropy theory of aging, which states that mutations that increase fitness early in life will be selected for, even when they have detrimental effects later in the life of the organism (Williams, 1957). Some authors go as far as to defend that all mutations with detrimental effects later in life are selected for their beneficial effects early in life. Some have

argued that a consequence of this reasoning is that any mutation extending longevity should decrease fitness of younger animals.

This has prompted the disposable soma theory of aging, stating that animals have limited resources to tap over the course of their lives. These resources are optimized for maintenance of the germline in liu of longevity, hence any condition that delays aging should negatively affect fertility (Kirkwood and Holliday 1979). In a simplified way, why spend valuable resources on protective enzymes that will allow the animal to live to 30 years when those resources would be better spent on reproduction, which ends at 15 years of age? This theory predicts that, any process that increases longevity leads to decreased fertility (Kirkwood and Holliday 1979). However, experimentally, this “trade-off” is not always present, thus suggesting that this theory is incomplete. Either the organism’s resources are not limited or there is room for further optimization of their allocation that would allow increases in longevity without a reproductive “trade-off”.

Currently, most evolutionary biologists tend to defend a mixed model involving the “mutation accumulation” and “antagonistic pleiotropy” theories. It is interesting to note that, although aging is a widespread phenomenon with shared characteristics throughout different species, evolutionary biologists predict it not to be genetically regulated (Kirkwood 2002). However, work done in the last 15 years has uncovered numerous lifespan-increasing mutations and has started to suggest that this view may be an oversimplification.

Another popular theory of aging is the rate of living theory. A century ago, Max Rubner noted that animals with higher metabolic rates often had shorter lifespans; this observation led to the rate-of-living theory of aging (Kirkwood 2002). In a very simplistic way, this is the “live fast, die young” theory of aging. The author proposed that all organisms possessed a certain amount of a “vital substance” that when used up would lead to death. For many years, the mechanistic connection between metabolic rate

and lifespan remained elusive. With the discovery of cellular oxidants (see section 1.3.1) however, this theory gained momentum as these provided the perfect connection between metabolic rate and longevity. Increased metabolic rates led to more oxidant-related damage which, in turn, decreased longevity. Although it is an attractive hypothesis due to its simple and intuitive nature, presently most scientists regard it as incomplete, mostly because the correlation between metabolic rate and lifespan is inconsistent across species. This is particularly true for birds and primates, which tend to live longer than predicted by their metabolic rates (Finkel and Holbrook 2000). Analysis of free radical formation in these species led to the finding that they have decreased production of toxic oxidants, suggesting that this could be the cause of their increased longevity. For a complete answer to our questions, theoretical models of aging need to be accompanied by experimental data. This experimental approach allows us to address another outstanding question in the field of aging research: How do we age? What are the causes of aging?

1.3 - Cellular Causes of Aging – *"How do we age?"*

Until now, we have discussed a non-specific decrease in function of living organisms, but on a more mechanistic note, exactly what causes the decrease in function observed with aging? If it is non-specific damage, why does it consistently affect a subset of processes?

1.3.1 - Oxidants and Aging

Presently, the most widely accepted culprits for age-associated decline in cellular and organismal function are reactive oxygen species (ROS), according to the free radical theory of aging (Harman 1956).

ROS, also called free radicals or oxygen radicals are highly reactive small molecules containing unpaired electrons. These molecules can react

with several organic molecules (nucleotides, proteins or lipids) and, in doing so, can cause considerable damage, impairing normal cellular function (Finkel and Holbrook 2000; Kirkinezos and Moraes 2001).

In the mid 1950s Denham Harnham introduced his free-radical theory of aging by speculating that cells generated endogenous oxygen radicals, which, in turn, resulted in a pattern of age-related cumulative damage.

Although, at first, the concept of endogenous oxidants was highly controversial, it gained momentum with the discovery of the first superoxide dismutase (SOD) enzyme (McCord and Fridovich 1969). Superoxide dismutases belong to a large family of important enzymes that detoxify ROS. SODs, in particular, are responsible for converting the superoxide anion ($^{\bullet}O_2^-$) into the more stable hydrogen peroxide (H_2O_2). H_2O_2, in turn, can be broken down into innocuous water (H_2O) and oxygen (O_2) by the enzymes catalase (Loew 1900) and glutathione peroxidase (Finkel and Holbrook 2000). However, if left unchecked, both superoxide and hydrogen peroxide molecules can lead to the production of the highly reactive hydroxyl radical (O_2^-), which is thought to be responsible for most cellular oxidative damage (Beckman and Ames 1998). Besides the aforementioned detoxifying enzymes, several other non-enzymatic, low molecular mass molecules can be found in cells that share their role in scavenging ROS. These include ascorbate, pyruvate, flavonoids, carotenoids and perhaps most importantly, glutathione (Finkel and Holbrook 2000).

ROS can damage three kinds of organic molecules: lipids, proteins and nucleotides. When reactive oxygen species react with cellular lipids, there can be significant decrease of membrane fluidity and generation of endoperoxides and unsaturated aldehydes. The latter are highly reactive and may act as mutagens, inactivate enzymes or operate as endogenous cross-linking agents (Beckman and Ames 1998). When ROS damage nucleotides, there may be formation of adducts of base and sugar groups,

single- and double-strand breaks in the nucleotide backbone, or cross-linking to other molecules. These altered nucleotides can eventually lead to mutation, DNA rearrangements or problems during transcription (Beckman and Ames 1998). Oxidation of proteins by oxygen radicals leads to formation of carbonyls, protein-protein cross-linking, peptide fragmentation and inactivation of proteins with iron-sulfur clusters (Beckman and Ames 1998). Overall, any of these interactions between ROS and biomolecules promote cellular dysfunction.

More importantly, however, there are several compelling lines of experimental evidence for the involvement of ROS in the aging process. Starting with the observation that, as animals age there is an accumulation of oxidant-damaged molecules in cells such as: protein carbonyls (Nystrom 2005), cross-linked lipid and protein species (lipofuscin) (Beckman and Ames 1998) and oxidant-damaged nucleotides, particularly mitochondrial DNA (mtDNA) (Esposito, Melov et al. 1999; Melov, Coskun et al. 1999). In addition, several age-related neurodegenerative diseases such as Parkinson's disease (Seaton, Cooper et al. 1997) or Alzheimer's disease (Mecocci, MacGarvey et al. 1994) show increased oxidative damage and mutations that increase steady-state ROS levels can lead to an early form of this diseases (Tu, Raju et al. 1996). Werner's syndrome, which is widely regarded to be a progeric (accelerated aging) process also leads to increases in protein carbonylation (Oliver, Ahn et al. 1987). It is also noteworthy that increasing atmospheric O_2 concentration in *C. elegans*, which increases oxidative stress, has been demonstrated to decrease longevity (Honda and Matsuo 1992). Moreover, It has been demonstrated in mice that the heterozygous state of *sod-2(+/-)* gene is enough to accelerate aging (Kokoszka, Coskun et al. 2001). However, this conclusion has been put into question by another study demonstrating that this decrease in *sod-2* levels, although increasing mitochondrial oxidative damage, does not affect longevity (Van Remmen, Ikeno et al. 2003). Overexpression of

superoxide dismutase leads to an increased lifespan in yeast (Harris, Bachler et al. 2005) and overexpression of SOD and catalase increase longevity of *D. melanogaster* (Orr and Sohal 1994; Sun and Tower 1999). Interestingly, flies can benefit from an increase in longevity when SOD is overexpressed only in the neurons arguing that this could be a rate-limiting tissue (Parkes, Elia et al. 1998), or there could be non-autonomous regulation originating in the neurons. Also, exposure to superoxide/catalase mimetics can extend C. elegans longevity up to 44% (Melov, Ravenscroft et al. 2000), although that work has been recently been put into question (Keaney, Matthijssens et al. 2004), interestingly, the latter authors saw a decrease in oxidative damage, but observed no effects on lifespan. Many long-lived *C. elegans* mutants show increased stress resistance which correlates with increased expression of *sod-3*, the nematode's SOD (Honda and Honda 1999; Feng, Bussiere et al. 2001; Henderson, Bonafe et al. 2006). In addition, certain short-lived *C. elegans* mitochondrial mutants, which show hallmarks of accelerated aging, have increased ROS-related damage (Ishii, Fujii et al. 1998; Kayser, Sedensky et al. 2004). However, the correlation between accelerated aging and increased ROS has been broken in several instances, which leads us to an important question:

Is oxidative stress resistance an absolute requirement for longevity? Is it, moreover, the sought-after mechanism of lifespan regulation? Experimental evidence suggests that the answer to these questions may not be a simple one. For instance, *chico* mutants in Drosophila are long-lived, but exhibit normal levels of resistance to oxidative stress (Clancy, Gems et al. 2001). In addition, mice knockout mutants for *SOD1*, *SOD2*, *SOD3* or *GPX1* (glutathione peroxidase) genes do not display a phenotype of accelerated aging (Reaume, Elliott et al. 1996; Melov, Schneider et al. 1998), despite increased levels or ROS. Finally, in *C. elegans*, expression of non-stress response genes is also influenced by insulin/IGF-1 signaling and some of them have been shown to contribute to the longevity of long-

lived mutants (Murphy et al. 2003, Lee, Kennedy et al. 2003). Thus, further research is required to determine the exact relationship between oxidative stress resistance and the regulation of longevity.

It is well established that during oxidative stress, accumulation of ROS leads to cellular damage. However, under physiological circumstances ROS have several "legitimate" cellular functions: they act as essential signaling molecules involved in wound healing (Sen 2003), recruitment of leukocytes during the adaptive immune response (Guzik, Korbut et al. 2003), regulation of aconitase activity (Gardner and Fridovich 1991; Gardner, Raineri et al. 1995; Irani, Xia et al. 1997) and regulation of the Ras pathway (Irani, Xia et al. 1997; Shibata, Branicky et al. 2003), mostly in the form of nitric oxide (NO·) and superoxide ($^{\bullet}O_2^-$). These newly-found roles of ROS in cellular signaling may have significant implications for their relationship to the aging process. Since the data suggests that there is an increase in free radicals in older organisms, it is tempting to speculate that many ROS-signaling dependent cellular processes will be altered with age, leading to a decrease in overall function of the organisms (Beckman and Ames 1998). This is an attractive hypothesis because potentially, smaller, more physiological amounts of "illegitimate" ROS could cause significant cellular miss-regulation and lead to aging.

1.3.2 - Mitochondria and Aging

As mentioned above, cells possess several enzymatic systems that allow them to produce "legitimate" ROS in a controlled fashion, to be used as regulatory molecules. Despite this fact, the majority of ROS is toxic and is generated as a by-product of the activity of one cellular organelle, the mitochondrion. Importantly, due to the proximity to the source, the primary targets of oxidant-related damage are also thought to be the mitochondria

themselves. In this way, mitochondria may be predicted to have a role in aging as the cell's main sources of ROS.

Mitochondria are complex cellular organelles consisting of two lipid membranes (outer and inner) with unique properties that separate two distinct compartments: the intermembrane space and the matrix (space inside the inner membrane) (Fig. 1).

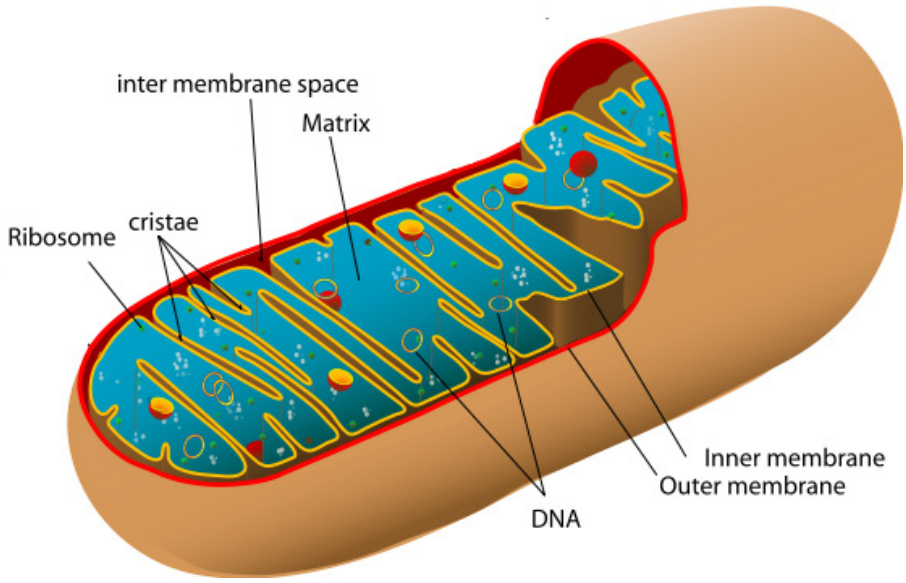


Figure 1 – Mitochondrial structure. Mitochondria have two distinct membranes (inner membrane and outer membrane) which separate two distinct compartments: matrix and inter membrane space. Mitochondria have their own genetic material, the nucleoid, which is circular and encodes their own set of translational machinery (ribosomes). These ribosomes are similar to bacterial ribosomes and distinct from eukaryotic ribosomes. This image was adapted from a wikicommons image.

According to the endosymbiotic theory (Wallin, 1923; Stocking 1959), mitochondria are thought to have arisen 1.7 (Feng, Cho et al. 1997; Emelyanov 2001) to 2 billion years ago (Feng, Cho et al. 1997; Emelyanov

2001) from an endosymbiotic proteobacteria that survived an endocytosis event. This pre-mitochondrial bacterium or proto-mitochondrion provided an evolutionary advantage to the host cell by performing aerobic respiration in cells that would have otherwise relied on less-efficient pathways of glycolysis and fermentation for energy (Sagan 1993). Although the majority of mitochondrial proteins are encoded in the nucleus, mitochondria contain their own genetic material (mtDNA) and translational machinery, reminiscent in many aspects of their prokaryotic evolutionary origins (Emelyanov 2003; O'Brien 2003). It is thought that over the millions of years since the appearance of eukaryotic organisms, much of the mitochondrial genetic material was transferred to the nucleus (Alberts et al., 2002). mtDNA molecules are usually circular, small (≈ 16 kb), attached to the inner mitochondrial membrane and code for ≈37 mitochondrial proteins essential for aerobic respiration (this number varies between species) (Alberts et al., 2002). This means that, although most mitochondrial proteins are encoded in nuclear DNA, cells with deficiencies in mtDNA are unable to perform mitochondrial respiration (see yeast “petites”, section 1.4.3).

Mitochondria serve several functions in eukaryotes: through unique metabolic reactions, they are the cell’s main source of energy, in the form of adenosine-triphosphate (ATP); they are essential in sequestering potentially toxic Ca2+ ions (Chan 2006); they serve an important role in apoptosis (Hengartner 2000); and they are necessary for steroid and heme synthesis (Chan 2006). Nevertheless, the most intensively studied and arguably the most important of mitochondrial functions is energy generation.

In the presence of oxygen, most cellular ATP is produced through aerobic respiration. This occurs in four distinct steps: the first one, glycolysis, which is not considered aerobic respiration *per se*, takes place in the cytosol. The next three steps occur in the mitochondria: pyruvate decarboxylation, the Krebs cycle and oxidative phosphorylation (Fig.2).

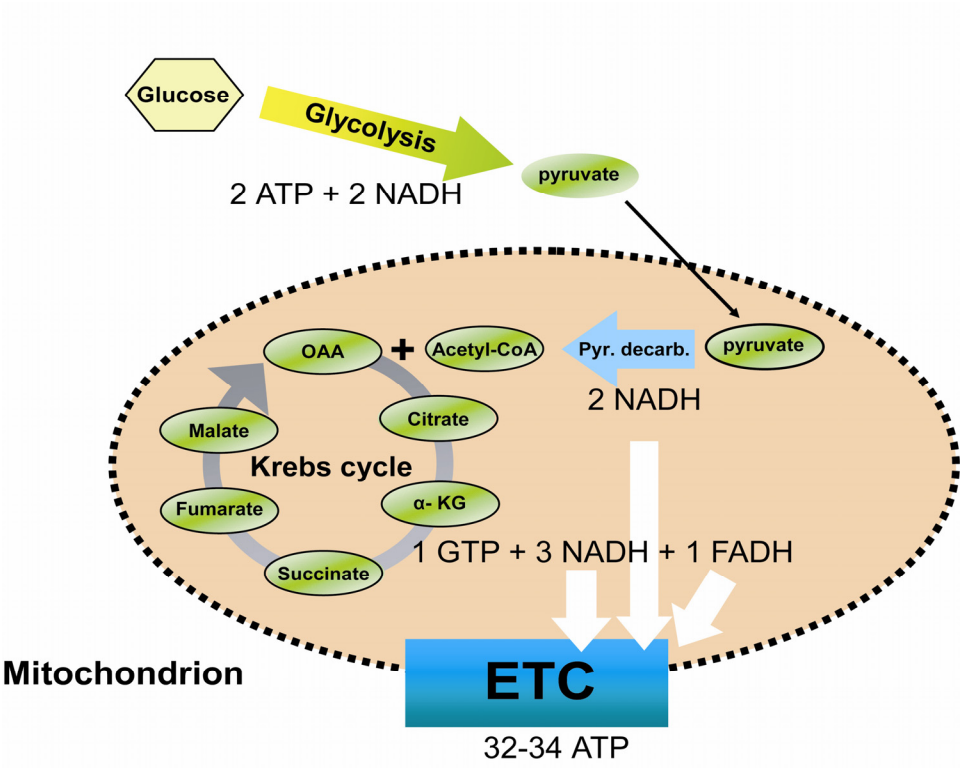


Figure 2 – Overview of aerobic respiration. ETC – electron transport chain; OAA – oxaloacetate; α-KG – α-ketoglutarate; Pyr. Decarb. – pyruvate decarboxilation. NADH produced during glycolysis may also be used by the ETC to produce ATP, however its transport into the mitochondria requires energy expenditure.

Glycolysis converts one molecule of glucose into two molecules of pyruvate, two molecules of ATP and two molecules of the reducing equivalent nicotinamide adenine dinucleotide (NADH). The resulting pyruvate is imported by the mitochondria where it is oxidized and combined with coenzyme A to produce two molecules of NADH and acetyl-CoA, which is the primary substrate of the tricarboxylic acid cycle (TCA) or Krebs cycle (Berg et al., 2002). In the mitochondrial matrix, the Krebs cycle converts acetyl-CoA to carbon dioxide (CO_2) and in this process there is production of: reducing equivalents, comprising three molecules of NADH and one molecule of flavin adenine dinucleotide (FADH); and, energy, in the

form of one GTP molecule that can be readily converted to ATP. The Krebs cycle is not only a central process in energy generation, but also produces many biochemical intermediates essential to other metabolic pathways such as glutamate, a key intermediate in amino acid metabolism that is generated from α-ketoglutarate (Butow and Avadhani 2004). Besides pyruvate decarboxylation, which yields acetyl-CoA, several other catabolic pathways provide intermediates for the Krebs cycle, especially when these intermediates are scarce. These are called anaplerotic pathways (Butow and Avadhani 2004). There are several different anaplerotic pathways, which can "feed" into different steps of the Krebs cycle (see chapter 3.4.1).

The last step in aerobic respiration, called oxidative phosphorylation, occurs in the inner mitochondrial membrane and converts reducing equivalents and oxygen into energy and water. These reducing equivalents, NADH and FADH, result mainly from the Krebs cycle. They are so named because they can donate electrons to other molecules, reducing them, and in the process donating redox energy contained in their molecular bonds. During oxidative phosphorylation, reducing equivalents donate electrons to the final acceptor O_2 in several steps via the electron transport chain (ETC) (Fig.3).

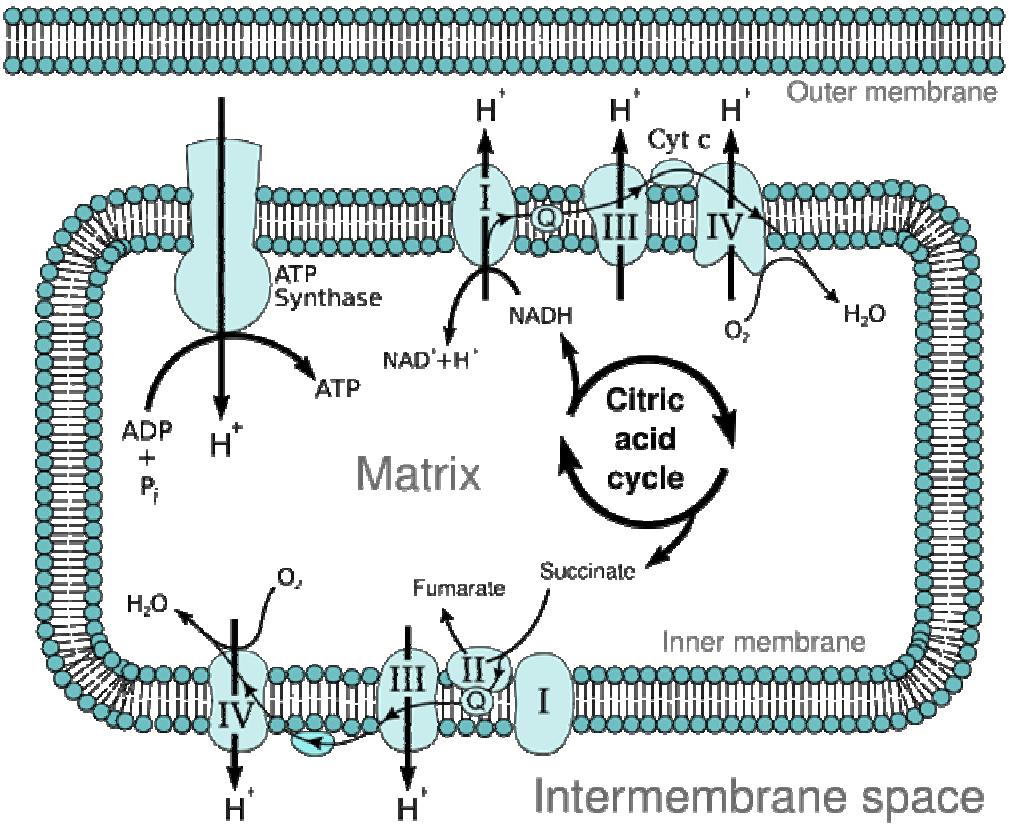


Figure 3 – Electron transport chain (ETC). Thin interconnected arrows depict flow of electrons. Q - electron carrier ubiquinone. Cyt c – electron carrier cytochrome c. H^+ - proton. P_i – phosphate. This image is adapted from a wikicommons image.

The electron transport chain comprises ubiquinone and cytochrome electron carriers and four protein complexes (complex I-IV) embedded in the inner mitochondrial membrane. These complexes transfer electrons sequentially to one another and to the final acceptor, molecular oxygen (O_2), producing water (H_20). In this process the redox energy generated during the electron flow is used by complex I, II and IV to pump protons (H+) into the intermembrane space, creating an electrochemical gradient

which is used by the highly conserved ATP synthase (sometimes called complex V) to generate ATP.

During oxidative phosphorylation, each O_2 molecule must accept two electrons to become fully reduced to H_2O, however, the process is imperfect and often only one electron is donated, leading to the formation of the above mentioned superoxide anions. The frequency of such events had been reported to be as high as 1-2% of all oxygen molecules (Boveris and Chance 1973), however presently it is believed that the frequency is 10-fold lower, at 0.1% (Imlay and Fridovich 1991). Nevertheless, 0.1% ROS generating events is still enough to qualify the mitochondria as the main source of cellular ROS. The formation of superoxides happens mostly at two discrete steps, complex I (NADH dehydrogenase) and Complex III (ubiquinone-cytochrome c reductase). Under normal conditions complex III is the main site of ROS formation (Turrens 1997). Studies have shown that the rate of flow of electrons during oxidative phosphorylation can influence the amount of ROS produced and many treatments that affect electron flow (complex activity inhibitors) produce increases in ROS production (Lenaz 2001). It is accepted that there is an optimal rate of electron flow in the ETC during oxidative phosphorylation, and both increases and decreases in this rate can lead to increased ROS production. Consistent with the idea that the mitochondria are a major source/target of oxidative stress, mitochondrially-dedicated superoxide dismutases and catalases have been identified (Fridovich 1995) and a component of the ETC, cytochrome c, acts as a powerful antioxidant (Beckman and Ames 1998). It is particularly interesting to note that several enzymes containing an active-site iron-sulfur cluster are deactivated in the presence of superoxide anions (Kuo, Mashino et al. 1987; Flint, Tuminello et al. 1993). Aconitase, which is an integral part of the Krebs cycle, has been experimentally shown to be inactivated in the presence of superoxide anions (Gardner and Fridovich 1991; Gardner, Raineri et al. 1995). Since the Krebs cycle is such a key metabolic process,

we can predict that the increase in ROS seen with age can also lead to increased metabolic dysregulation, with grave and pleiotropic cellular consequences.

According to the free radical theory of aging, oxidant-related damage is an important player in the aging process, and since mitochondria are the cells' main ROS generators, they too are likely to have an important role in aging. Furthermore, it follows that, since the mitochondria are the main source of ROS they are also extremely susceptible to oxidative damage, by proximity. Experimental evidence supports this, as there is decreased mitochondrial function with age, in the form of damaged mtDNA (Melov, Lithgow et al. 1995). The mitochondrial genome contains 13 subunits of complexes I, III and IV. Mutations in these genes lead to decreased mitochondrial function and increased ROS generation (Beckman and Ames 1998). This is thought to create a positive feedback loop or "vicious cycle" where oxidant-damaged mitochondria produce more oxygen radicals, which in turn exacerbate their own damage (Finkel and Holbrook 2000; Kirkinezos and Moraes 2001). Consistent with this theory, large deletions of the mitochondrial genome (ΔmtDNA) accumulate exponentially in aging humans (Cortopassi and Arnheim 1990), *C. elegans* (Melov, Lithgow et al. 1995), mice (Chung, Weindruch et al. 1994; Tanhauser and Laipis 1995), rats (Edris, Burgett et al. 1994) and rhesus monkeys (Lee, Chung et al. 1993). In addition, increased frequencies of ΔmtDNA are found in age-associated neurodegenerative diseases such as Huntington's and Alzheimer's disease. (Beckman and Ames 1998). Moreover, point mutations accumulate in mtDNA with age (Michikawa, Mazzucchelli et al. 1999; Wang, Michikawa et al. 2001). It has been demonstrated that oxidative damage is more extensive and persists for longer in mtDNA than in nuclear DNA (Yakes and Van Houten 1997), potentially due to less efficient DNA protection mechanisms in the mitochondria. Nevertheless, the "mtDNA mutation accumulation" theory of aging, as it is known, remains to

be proven conclusively. It is still unclear whether the increase in frequency of ΔmtDNA and mtDNA point mutations is causally related to increased oxidative damage, or is merely a biomarker of aging.

Further emphasizing the connection between mitochondria and aging, work done in *C. elegans* and *S. cerevisiae* has uncovered several mutants with impaired mitochondrial function that have altered lifespans. Some of these mutants have shortened lifespans (Ishii, Fujii et al. 1998; Kayser, Sedensky et al. 2004), whereas others, interestingly, live considerably longer than their wild-type counterparts (Wong, Boutis et al. 1995; Kirchman, Kim et al. 1999; Epstein, Waddle et al. 2001; Feng, Bussiere et al. 2001; Traven, Wong et al. 2001; Dillin, Hsu et al. 2002; Lee, Lee et al. 2003). These fascinating long-lived mitochondrial mutants and the regulation of their phenotypes will be the focus of this PhD thesis (for a more detailed discussion of these mutants, please see section 1.4.3).

1.3.3 - Other potential causes of aging

This section presents an overview of other cellular processes potentially involved in aging that are beyond the scope of this thesis.

The discovery of telomeres (reviewed in Blackburn 1990), telomerase (Blackburn, Greider et al. 1989) and their role in cellular senescence pointed to an obvious connection between telomeres and aging (Kruk, Rampino et al. 1995). However, although this is an area of intense research, the extent of said connection remains unknown. During each replication event, chromosomes lose DNA at the tips of their chromosomes. As they undergo more replication events, chromosomes tend to become shorter so, in order to avoid losing valuable coding chromosomal DNA, short repetitive non-coding DNA sequences are added to their tips, these are called telomeres. Telomeres act as “buffers” during replication, by shortening themselves with each division, avoiding loss of important genetic

information. But of course, even telomeres would eventually “wear down”, so in order to re-elongate them to their original length, an enzyme called telomerase is required (Blackburn 1990). Telomeres also prevent chromosome fusions that can lead to cancer (DePinho 2000). As a result, when telomeres become too short, possibly in order to avoid becoming cancerous, cells either stop dividing and enter a senescent state, or self-destruct through apoptosis (DePinho 2000). Because of this, cells have a limited number of divisions they can go through, linked to telomeric length, the Hayflick limit (Hayflick 1965). In this sense, telomeres can act as biological “clocks”. This idea generated much interest in the aging research community, but, despite this, the effect of telomeres and senescence on aging remains controversial (Campisi 2005). On the one hand, many lines of evidence connect ROS damage to increased senescence and telomere shortening (Chen and Ames 1994; von Zglinicki, Saretzki et al. 1995; Oexle and Zwirner 1997; Lee, Fenster et al. 1999; Passos, Saretzki et al. 2007), providing a potential role for telomeres in aging. On the other hand, experiments attempting to establish a causal link between telomere length, senescence and aging have come up with unclear results (Gonzalez-Suarez, Geserick et al. 2005). Overall, the relationship between cancer, aging and telomeres is complex and needs further study.

The ability to guarantee proper protein folding is essential for cellular function and organismal viability (Balch, Morimoto et al. 2008). In cells undergoing division, damaged and oxidized proteins are retained in mother cells, allowing daughter cells a proteomic “fresh start”. However, in post-mitotic tissues, cells that are not actively dividing, such as neurons, need more sophisticated mechanisms to keep their proteome healthy. These mechanisms range from protein chaperones to protein degradation machinery. Although many proteins fold into their three-dimensional structures spontaneously, others require assistance from a sophisticated array of enzymes called chaperones. Occasionally, despite these robust

chaperoning systems and quality control mechanisms that assure protein integrity, polypeptides still fold incorrectly and must be dealt with to avoid additional cellular damage. These misfolded or damaged proteins are targeted for degradation and degraded by specialized cellular machinery (Hebert and Molinari 2007), called proteasomes. However, sometimes, misfolded proteins may "escape" degradation by the proteasome. When this happens, it can lead to aggregation of misfolded proteins and, subsequently, to a "clogging" of the degradation machinery. This, in turn, can destabilize the cellular proteome leading to aggregation of other unrelated polypeptides (Gidalevitz, Ben-Zvi et al. 2006) and, in severe cases, disease. These diseases are collectively known as protein-aggregation diseases or conformational diseases (Kopito and Ron 2000). Among these conformational diseases are human neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and Huntington's disease. It is understood that all these diseases involve aggregation of specific proteins, however, the specific process by which disease develops remains unknown. It is currently accepted that the likely culprits are intermediate aggregative oligomeric structures sometimes called protofibrils (Caughey and Lansbury 2003; Haass and Selkoe 2007). It was initially thought that the easily observable larger cellular aggregates were responsible for impairing cellular function (Forloni 1996), but they are now believed to serve a protective purpose (Arrasate, Mitra et al. 2004). How is this related to aging? The major risk factor for protein aggregation diseases is age (Amaducci and Tesco 1994). Individuals containing disease-linked mutations manifest the disease in their fifth decade and spontaneous aggregation diseases appear in the seventh decade (Amaducci and Tesco 1994). Also, *C. elegans* and mouse transgenic models expressing aggregation-prone proteins at a constant level only show aggregation at later stages in life (Jankowsky, Savonenko et al. 2002; Brignull, Morley et al. 2007). These findings support the notion that the

cell's ability to cope with protein misfolding stress is altered with age. Still, the argument could be made that there is a constant accumulation of misfolded proteins irrespective of the organism's age, and that some critical mass must be obtained before disease symptoms occur, thus separating disease from aging. Interestingly, recent studies contradict this hypothesis. In *C. elegans*, time of onset of aggregation can be delayed by approaches that increase longevity, such as inhibiting insulin pathway activity (Morley, Brignull et al. 2002; Hsu, Murphy et al. 2003). This is a strong argument for a connection between protein aggregation and aging. There are two possible explanations for this observation: On one hand, some of the factors contributing for aging could also be causing protein aggregation, it is tempting to speculate that as organisms age, there is a decrease in quality of protein folding mechanisms. On the other hand, protein aggregation itself could be causal to aging, as it is already clear that increases in misfolded proteins impair organismal function (Hsu, Murphy et al. 2003). In all likelihood both of these are accurate to some extent.

Accumulation of somatic mutations was proposed as another possible cause of aging (Miquel 1992; Bohr and Anson 1995; Evans, Burbach et al. 1995; Morley 1995; von Zglinicki, Nilsson et al. 1995). If we consider cancer to be a symptom of aging, then surely mutations play an important role in the aging process (Ames, Gold et al. 1995). However, the question remains: do somatic mutations play a role in other phenotypes of aging? A study showed an age-dependent increase in mutations in a *lac*Z transgene in mouse liver. However, this argument is weakened by the observation that mice expressing this same transgene in their post-mitotic brain showed no significant increase in mutations (Dolle, Giese et al. 1997). This suggests that, although more prominent with age, somatic mutations have little effect on an important hallmark of aging organisms, which is age-related neurodegeneration. Also, the liver mutations were not dramatic and are unlikely *per se* to have a large effect in the function of the organism (Warner

and Johnson 1997). So, what evidence is there supporting somatic mutations as a potential cause of aging? A compelling line of evidence is the high correlation between DNA repair ability and lifespan (Hart and Setlow 1974; Cortopassi and Wang 1996). This is indicative that increased DNA repair may play a role in increasing longevity. However, it has been demonstrated that effective DNA repair is necessary but not sufficient to increase longevity (Hart and Setlow 1974; Cortopassi and Wang 1996). The role of somatic mutations and DNA repair mechanisms in aging remains controversial. Clearly, organisms need an effective DNA repair system to avoid cancer and realize their longevity potential. The question is whether this DNA repair system and somatic mutations play a role in normal aging.

1.4 - Modulation of Aging – *"Can we age more gracefully?"*

Is aging no more than passive decay of cellular function, much like "wear and tear" of an old car? Alternatively, are there biological mechanisms regulating aging and if so, can we manipulate them to increase longevity? These are key questions in aging research and ones that we can begin to address due to work done in the last 20 years. The discovery of genetic manipulations capable of extending longevity has pushed the aging field from a more descriptive scientific endeavor to one of mechanistic, hypothesis-driven work. The discovery that single gene mutations in the same genes can extend longevity across different model organisms has given much strength to the argument that aging is regulated in an evolutionarily conserved way. This, in turn, has fueled the notion that potentially, human aging and the onset of age-related disease are amenable to manipulation. The possibility of aging-related therapeutics has generated much excitement in the field and has fueled the emergence of several privately owned companies. Since the discovery, over 20 years ago, that genetic manipulations in *C. elegans* could extend longevity of

mutants relative to wild-type (Klass 1983) many more gerontogenes (genes affecting longevity) have been identified in worms (Kenyon, Chang et al. 1993; Lee, Lee et al. 2003; Hamilton, Dong et al. 2005; Hansen, Hsu et al. 2005). Despite the large number of genes, they can generally be broadly grouped into one of three distinct pathways influencing longevity: reduced food intake (dietary restriction or DR), reduced insulin signaling or other conditions that activate the DAF-16/FOXO transcription factor, or impaired mitochondrial function (which is the main focus of this thesis). These three pathways have been shown to be conserved in *C. elegans* and rodents, suggesting the presence of an evolutionarily conserved mechanism for regulation of lifespan.

Evidence for the independence of these pathways comes from three groups of observations: First, double-mutant analysis shows that the effects of these pathways are additive to each other (Lakowski and Hekimi 1998; Dillin, Hsu et al. 2002), suggesting they are not acting through the same process. Second, the temporal requirements of these three pathways are different: loss of insulin signaling during adulthood regulates longevity (Dillin, Crawford et al. 2002); mitochondrial impairment has to occur during development to increase longevity (Dillin, Hsu et al. 2002); and DR can act at any timepoint throughout the animals lifespan to regulate longevity, at least in flies (Mair, Goymer et al. 2003). The third set of observations refer to transcription factors that are absolutely required for lifespan extension to occur in one pathway but are dispensable for longevity in other pathways (Lakowski and Hekimi 1998; Houthoofd, Braeckman et al. 2003; Houthoofd, Braeckman et al. 2005). Besides increasing lifespan, manipulation of these three independent pathways leads to overlapping phenotypes such as: resistance to oxidative stress, thermal stress and DNA damage stress (Hekimi and Guarente 2003). This prompts the question of whether they are merely different upstream regulatory pathways affecting overlapping sets of similar downstream effectors. Are there different ways to activate a similar

response? In order to address this question we need to understand each of these pathways in more detail. Of the three pathways I will provide greater background on the mitochondrial inhibition pathway of aging, since it is the main point of this thesis.

1.4.1 - Dietary Restriction (DR)

Over 70 years ago Mcay et al. reported that decreasing food intake increased lifespan in rats (McCay, Crowell et al. 1989). Since then, this finding has been reproduced in several typical model organisms such as: mice (Mair and Dillin 2008), nematodes (*C. elegans*) (Klass 1977; Houthoofd, Braeckman et al. 2003), budding yeast (*S. cerevisiae*) (Jiang, Jaruga et al. 2000), fruit flies (*D. melanogaster*) (Chapman and Partridge 1996) and in some more atypical model organisms such as water striders, grasshoppers, water fleas, fish, hamsters, dogs and spiders (Mair and Dillin 2008). Studies on rhesus monkeys (Hansen and Bodkin 1993; Kemnitz, Weindruch et al. 1993) and squirrel monkeys (Ingram, Cutler et al. 1990) are ongoing and results are promising if still preliminary. In spite of the amount of time elapsed since its discovery and its impressive evolutionary conservation, the mechanism by which DR acts remains elusive. For many years, the field of DR research was lacking molecular studies, probably because most studies were done in rodents, which are not as amenable to genetic manipulation as some of the invertebrate model organisms.

Why would decreasing food intake increase longevity? It has been proposed that DR may have been evolutionarily selected for, since, during low-food situations it delays the onset of reproduction and increases longevity. This allows the animals to hold-off committing to the high-energy endeavor of reproduction until resources are available (Holliday 1989). This increase in energy availability would increase the organism's chances of reproducing successfully and of its offspring surviving.

An argument for DR's evolutionary conservation is that dietary-restricted animals share multiple phenotypes across different species in addition to the increased longevity. Mainly, as mentioned before, a decrease in overall reproductive potential and a delay in onset of reproduction have been observed in fruit flies (Mair and Dillin 2008), worms (Klass 1977; Bishop and Guarente 2007; Bishop and Guarente 2007) and rats (Mair and Dillin 2008). Also, DR in model organisms has been shown to delay onset of age-related diseases such as: autoimmune disease, osteoporosis, cataracts, neurodegenerative diseases, diabetes and several forms of cancer (for a more detailed review see chapter V of Masoro, 2002). This means that dietary-restricted animals do not live longer as frail aged individuals, but are instead maintained in a healthier state until later in life.

DR is defined as a significant decrease in caloric intake that does not constitute starvation, usually 30-40% lower than fully fed. DR has been modeled in many different organisms and in many different ways: from decreasing all food availability (Klass 1977; Fabrizio and Longo 2003), to specifically altering the animal's diet (Zimmerman, Malloy et al. 2003; Miller, Buehner et al. 2005), to mutations that decrease food intake (Lakowski and Hekimi 1998), (for a detailed listing of all, please see (Mair and Dillin 2008)). As a consequence of all these different methodologies, results can vary significantly between researchers, which has considerably slowed the field. The complexity and variability of the response to CR, when administered under different conditions, was unexpected. It will be very interesting to learn its basis at the molecular level.

Despite this, progress has been made in understanding the regulatory mechanisms of DR. The first potential regulator of DR was discovered in budding yeast, it is the NAD^+-dependent histone deacetylase *SIR2*. Guarente et al. showed that a mutant yeast strain null for *SIR2* were not long-lived upon reduction of glucose in the medium, a condition know to induce DR (Lin, Defossez et al. 2000). Since then, researchers have shown

that overexpression of Sir2p in yeast (Lin, Defossez et al. 2000), worms (Tissenbaum and Guarente 2001) and flies (Rogina and Helfand 2004) extends longevity, which clearly implicates this conserved family of proteins (sirtuins) in aging. However, new lines of evidence have put the initial yeast findings into question (Kaeberlein, Kirkland et al. 2004; Kaeberlein and Powers 2007), and this, in turn, has significantly weakened the connection between DR and sirtuins. Consequently, at this point, the connection between sirtuins and DR remains unproven. Another potential modulator of lifespan through DR is the target of rapamycin (TOR) pathway (Mair and Dillin 2008). The TOR nutrient sensing pathway has been implicated in longevity in several model organisms (Schieke and Finkel 2006), and is the best candidate for a conserved regulator of DR, found so far. Recently, two transcription factors *skn-1*, which is induced in response to oxidative stress (Bishop and Guarente 2007) and *pha-4*, a forkhead transcription factor (Panowski, Wolff et al. 2007) have been identified as necessary for DR-mediated increased longevity in *C. elegans*. These are promising molecules which may allow genetic probing of DR regulatory pathways. However, it remains to be shown whether their effects are conserved across different species. Interestingly, recent data has pointed to a role of autophagy in DR-mediated increased longevity (Jia and Levine 2007; Hansen, Chandra et al. 2008), this is suggestive that recycling of cellular components has an important role in DR and longevity in *C. elegans*

1.4.2 - Insulin/IGF-1 (IIS) dependent regulation of aging

The best characterized pathway regulating longevity is the Insulin/IGF-1 (IIS) dependent regulation of aging. This pathway was originally identified in *C. elegans* (Friedman and Johnson 1988; Kenyon, Chang et al. 1993) where it has been studied to the greatest extent. More recent work, points to evolutionary conservation in fruit flies (Tatar, Kopelman et al. 2001) and

mice (Bluher, Kahn et al. 2003; Holzenberger, Dupont et al. 2003), making this an important therapeutic target for longevity modulation in humans (Bartke 2008; Suh, Atzmon et al. 2008).

IIS is an important nutrient-sensing pathway that coordinates processes such as development, fat metabolism, growth and reproductive maturation. As such, this pathway is a good candidate for regulating longevity in response to energy availability, much like what was proposed earlier for DR. A possible model is that less energy availability leads animals to post-pone their reproductive maturation in order to increase likelihood of viable progeny (Wolff and Dillin 2006). This is consistent with the function of IIS pathway in worms, in that it regulates a hibernation-like state (the dauer diapause) that can occur during development if resources are scarce (Riddle, Swanson et al. 1981). Animals in this dauer stage are very resistant to thermal (Lithgow, White et al. 1995) and oxidative stresses (Honda and Honda 1999) and are considerably long-lived. It is all-together possible that the long lived *C. elegans* IIS mutants are activating and benefiting from the same protective mechanisms that allow dauers to live long, but without the hibernation phenotypes. However, the time during the animals' life when the IIS pathway controls reproduction is different from the time when it affects aging (Dillin, Crawford et al. 2002). Also, mutants with different alleles have normal reproduction profiles. These are a strong arguments against a causal connection between rebroduction and aging .

In *C. elegans* the IIS pathway has been thoroughly dissected and most of its components identified, for a complete review please see (Kenyon 2005). The most representative long-lived IIS mutant in *C. elegans* is *daf-2(-)*. DAF-2 is the worm's sole insulin/IGF-1 receptor. *C. elegans* IIS pathway converges on the forkhead transcription factor *daf-16*, meaning that, in the absence of *daf-16*, *daf-2* mutants no longer have increased lifespan (Kenyon, Chang et al. 1993).

Microrray studies of *daf-2(-)*, *daf-16(-)* and *daf-2(-)*; *daf-16(-)* mutants showed that the DAF-16 forkhead transcription factor modulates expression of several downstream genes affecting longevity (Murphy, et al. 2003). Interestingly, individual knock downs of each these genes in long-lived *daf-2* mutants were insufficient to completely suppress longevity. This suggests that the total lifespan observed in long-lived *daf-2* mutants is a result of small cumulative effects from multiple genes downstream of *daf-16*. Therefore, based on this finding, even though aging is regulated by a few key pathways, many downstream genes affect longevity.

Mutations in *daf-2* lead to significant metabolic remodeling of the organisms as can be ascertained from gene expression studies (Murphy, et al. 2003). One interesting change is an increase in expression of enzymes necessary for the glyoxylate cycle (Murphy, et al. 2003). The glyoxylate cycle is an anaplerotic pathway that feeds intermediates into the Krebs cycle, thus allowing production of energy from stored fats. Another characteristic of *daf-2* mutants is an increase in stress resistance (Larsen 1993; Vanfleteren 1993; Adachi, Fujiwara et al. 1998), which is accompanied by an increase in expression of several stress protective genes under control of *daf-16* (Vanfleteren and De Vreese 1995; Honda and Honda 1999; Murphy, et al. 2003; Lin, Hsin et al. 2001). It is interesting to note that, there is a high (albeit imperfect) correlation between longevity and stress resistance in most long-lived mutants. This holds true for mutants that live long through DR, IIS inhibition or mitochondrial impairment.

In *C. elegans* germline removal also affects longevity through the *daf-16* forkhead transcription factor (Hsin and Kenyon 1999), and for that reason this pathway is discussed in this section. However, this pathway could potentially be insulin/IGF-1 independent. Animals missing their germline are long-lived in a *daf-16* dependent fashion. However, this lifespan is synergistic with *daf-2*, which suggests that these are partially independent

pathways. On the other hand, if these animals lack their entire gonad they do not exhibit an extended lifespan except when combined with some classes of *daf-2* mutants, raising the possibility of partial mediation of this extension by the IIS (Hsin and Kenyon 1999). These findings support a connection between longevity and reproduction, although the nature of this connection is still unclear.

There is also significant evidence that sensory neurons affect longevity (Apfeld and Kenyon 1999; Alcedo and Kenyon 2004) in an IIS partially-dependent fashion. When sensory neuron function is impaired, worms live longer (Apfeld and Kenyon 1999). Certain subsets of sensory neurons can affect longevity in opposite ways. Different gustatory neurons can both extend and shorten longevity in a IIS dependent fashion (Alcedo and Kenyon 2004). Olfactory neurons, on the other hand, can affect lifespan in a distinct pathway that is connected to the reproductive system (Alcedo and Kenyon 2004) and not dependent on insulin signaling.

1.4.3 - Long-lived mitochondrial mutants*

*(For a summarized version of this section please see section 3.2)

In section 1.3.2 (Mitochondria and Aging), we showed several lines of evidence linking mitochondria to longevity. One of the most compelling arguments is that in budding yeast, nematodes and mice, mutations that affect normal mitochondrial function can lead to increased longevity. This observation in itself is somewhat paradoxical. Since mitochondria and mitochondrial respiration are vital processes in cellular metabolism, it is unexpected that from their impairment organisms could reap longevity benefits.

In yeast, this phenomenon was initially observed through the usage of “petite” strains, these are strains that lack mitochondrial DNA, or fully functional mitochondria (Kirchman, Kim et al. 1999). These can occur

naturally or be induced through short exposure to ethidium bromide, which preferentially inhibits replication of small genomes such as the mitochondrial genome (Miceli and Jazwinski 2005). Researchers observed that some spontaneous “petite” strains actually had increased longevity. Longevity was measured as number of daughter buds per yeast cell, also called replicative age (Mortimer and Johnston 1959; Muller, Zimmermann et al. 1980), as opposed to chronological age. In this initial work (Kirchman, Kim et al. 1999), it is proposed that lack of respiration *per se* is not the cause of the increased longevity. When cells are grown in media containing glucose as the carbon source, which under normal circumstances represses respiration, they still see an increase in longevity. The authors go on to demonstrate that a retrograde response (or retrograde regulation) was responsible for the increase in longevity observed in yeast “petites” (Kirchman, Kim et al. 1999). Yeast cells with partial or total loss of mtDNA (Parikh, Morgan et al. 1987), present altered gene expression profiles. This response has been labeled retrograde response (Liao and Butow 1993). “Retrograde response” is the general term for mitochondrial signaling and is broadly defined as cellular responses to change in the functional state of mitochondria. Implicit in this is that this flow of information is opposite to the normal anterograde flow of information and proteins form the nucleus and cytoplasm into the mitochondria. It is unclear exactly how this flow of information occurs, but a few players have already been identified. These are *RTG1*, *RTG2* and *RTG3* (Chelstowska and Butow 1995), and mutation of these genes prevents yeast “petites” from living long (Kirchman, Kim et al. 1999). *RTG1* and *RTG3* encode basic helix-loop-helix leucine zipper type transcription factors. They heterodimerize and bind to the promoter region of target genes (Liu and Butow 2006). Rtg2p is a cytoplasmic protein with an N-terminal ATP binding domain belonging to the actin/Hsp70/sugar kinase superfamily (Liu and Butow 2006). It is known that Rtg2p is essential for nuclear translocation of the Rtg1p/Rtg3p complex and that the ATP

binding domain is essential for this, but the mechanism remains unclear (Liu, Sekito et al. 2003). One of the initial markers for the retrograde response was increased expression of the gene *CIT2*, which encodes a citrate synthase (Liao, Small et al. 1991), this suggested that there might be some form of metabolic response to mitochondrial impairment.

In order to better understand the underlying cellular processes, two genome-wide expression microarray studies comparing respiratory-competent to respiratory-deficient ("petites") yeast cells, were completed (Epstein, Waddle et al. 2001; Traven, Wong et al. 2001). These studies showed significant metabolic and stress response remodeling of the cells (Fig. 13). Genes involved in glycolysis were up-regulated (Epstein, Waddle et al. 2001; Traven, Wong et al. 2001). One possible explanation for this is that, since yeast "petites" are unable to perform respiration, they are likely to become increasingly dependent on glycolysis as their main source of ATP. Also, there was an increase in expression of many genes either directly involved in the Krebs cycle, or in anaplerotic pathways linked to it (Epstein, Waddle et al. 2001; Traven, Wong et al. 2001). A potential reason for this up-regulation of the Krebs cycle is that respiration-deficient "petites" may be compensating for a depletion of cellular glutamate (Fig. 13). Glutamate is central to amino acid metabolism and is produced from an intermediate of the Krebs cycle, α-ketoglutarate. As a consequence of this, treatments that impair the Krebs cycle lead to, among other things, depletion of cellular glutamate (Butow and Avadhani 2004). Importantly, the Krebs cycle enzyme succinate dehydrogenase is an integral part of the electron transport chain (ETC). In the process of converting succinate to fumarate, this enzyme donates a pair of electrons to its coenzyme flavin adenine dinucleotide (FADH) reducing it. These electrons go on to enter the ETC through oxidative phosphorylation. However, if the ETC is non-functional, there is no way to re-oxidize the coenzyme FAD to its electron accepting state. This, in turn means that succinate dehydrogenase is

inhibited and the Krebs cycle cannot be completed. In summary, inhibition of mitochondrial respiration and electron flow through the ETC, leads to interruption of the Krebs cycle and accumulation of succinate. This inability of the Krebs cycle to be completed, in turn, leads to a decrease in α-ketoglutarate availability and to a depletion of cellular glutamate (Butow and Avadhani 2004). Hence, it is likely that cells are increasing expression of Krebs cycle enzymes and anaplerotic pathways in order to keep the cycle flowing, if only partially to allow for production of glutamate.

These two microarray analyses also showed very significant up-regulation of glyoxylate cycle enzymes. Glyoxylate cycle is an important anaplerotic pathway. It bypasses the succinate-to-fumarate step of the Krebs cycle through the formation of glyoxylate, and eventually leads to the formation of pyruvate, which can be converted into alpha-ketoglutarate. This metabolic process occurs in the peroxisomes (Fig. 13). Another class of gene that was seen to have increased expression in these long-lived yeast “petites” was the stress response class(Epstein, Waddle et al. 2001; Traven, Wong et al. 2001). These stress response genes encoded mitochondrial chaperones (Traven, Wong et al. 2001), heat shock proteins (Epstein, Waddle et al. 2001; Traven, Wong et al. 2001) and stress response transcription factors (Epstein, Waddle et al. 2001; Traven, Wong et al. 2001). It is interesting to note that there was one inconsistenciy between the two studies, namely Traven et al. found increased expression of numerous genes encoding mitochondrial proteins, strongly suggesting that the “petites” were trying to compensate for the deficient mitochondrial respiration. On the other hand, Epstein et al. saw no change in expression levels of these genes. This can potentially be explained by differences in the strains used for each study (Butow and Avadhani 2004).

It is interesting to note that the retrograde response is likely evolutionarily conserved, since it has also been described in mammalian cells with depleted mtDNA (Miceli and Jazwinski 2005). However, it is

unclear whether its role in longevity is specific to yeast, or whether retrograde responses can be important processes in regulating longevity of other, multicellular, organisms. This question is addressed in chapter III of this thesis.

As important as the yeast work has been in understanding this complex relationship between mitochondrial mutants and aging, much work on this subject has also been conducted in *C. elegans*. In fact, the first long-lived mitochondrial mutant was identified in the nematodes, *clk-1* (Wong, Boutis et al. 1995; Lakowski and Hekimi 1996). These mutants were identified as long-lived *C. elegans* mutants (Lakowski and Hekimi 1996) with a particular set of phenotypes, namely, that their rates of living were slower than wild type's. These animals took longer to develop to adulthood, and generally moved slowly when compared to their wild-type counterparts (Wong, Boutis et al. 1995). *clk-1* mutants showed decreased rates of pharyngeal pumping, defecation and swimming. This gene was later identified to encode a mitochondrial hydroxylase necessary for synthesis of ubiquinone, a component of the ETC (Ewbank, Barnes et al. 1997). Ubiquinone (Q_9; the subscript refers to the number of isoprene units) is a prenylated benzoquinone lipid required for shuttling electrons from complexes I and II to complex III during respiration. In yeast, mutations in *COQ7*, the yeast homologue of *clk-1*, lead to a loss of viability in the presence of a non-fermentable carbon source (Jonassen, Marbois et al. 1996). Interestingly, despite the likely detrimental effect of this mutation on mitochondrial respiration, *C. elegans clk-1* mutants are not only viable but also have wild-type ATP levels (Braeckman, Houthoofd et al. 1999) and close to wild-type rates of respiration (Felkai, Ewbank et al. 1999). The reason for this difference between *C. elegans* and *S. cerevisiae* is that *clk-1* mutants can compensate for their lack of Q_9 with another ubiquinone isoform they obtain from their diet, Q_8 (Jonassen, Larsen et al. 2001; Larsen and Clarke 2002). Like all mitochondrial mutants discovered so far, *clk-1(-)* animals' longevity

seems independent of DR or the IIS pathways for controlling longevity, which sets them up as a *bona fide* independent pathway for longevity regulation. Importantly, *clk-1*'s role in longevity is likely conserved, since heterozygous mice with reduced levels of *Mclk-1* live longer than controls (Stepanyan, Hughes et al. 2006).

Another long-lived *C. elegans* mitochondrial mutant, *isp-1*, was identified in a mutagenesis screen and was found to have several phenotypes in common with *clk-1(-)* animals (Feng, Bussiere et al. 2001). These *isp-1(-)* animals harbor a point mutation in an iron-sulfur protein located in complex III of the ETC (Feng, Bussiere et al. 2001). *isp-1* mutants exhibit extraordinarily decreased rates of living and delayed development to adulthood (Feng, Bussiere et al. 2001). In many respects these mutants have similar, although more dramatic, phenotypes than *clk-1*. However, they differ in a significant trait; *isp-1* mutants have a significant decrease in mitochondrial respiration as measured by oxygen consumption (Feng, Bussiere et al. 2001). Also, as a potential mechanism for their increased longevity these mutants are resistant to oxidative damage and increase expression of antioxidant enzymes (Feng, Bussiere et al. 2001).

Recent studies in *C. elegans* have demonstrated that extreme inhibition of mitochondrial function can have detrimental effects and only moderate mitochondrial defects lead to increased longevity (Rea, Ventura et al. 2007). On the other hand, with the advent of RNA interference (RNAi) methodology (Fire, Xu et al. 1998; Timmons, Court et al. 2001) it became possible to screen whole genome libraries for gene knockdowns that would extend longevity. The advantage of such approaches is two-fold: a) knockdowns are more likely to be viable than knockouts; and b) since the RNAi exposure is achieved through feeding, the animals go through their embryonic development with intact function of the gene, thus bypassing a sensitive developmental stage. Two separate RNAi screens for genes that when knocked down increased longevity of *C. elegans* identified a very

significant role for mitochondrial respiration in lifespan (Dillin, Hsu et al. 2002; Lee, Lee et al. 2003). These studies showed that knocking down several different components of the ETC significantly increased longevity and decreased rates of living (Dillin, Hsu et al. 2002; Lee, Lee et al. 2003). These phenotypes are similar to the ones observed in *clk-1* and *isp-1* mutants. These long-lived mitochondrial RNAi knockdowns were also smaller than wild type animals and had decreased ATP production (Dillin, Hsu et al. 2002).

Importantly, when looking at long-lived mitochondrial mutants in *C. elegans*, even though they share several phenotypes, there is an important distinction between the ones that do not affect respiration and energy production, *clk-1* mutants; and the ones that do, *isp-1* mutants and RNAi-induced mitochondrial knockdowns. This has prompted a long-standing question in the field, which is: How similar are these long-lived mitochondrial mutants to each other? Since they share so many phenotypes the simplest explanation is that they share a common mechanism in regulating their increased longevities. If so, however, why do they have different phenotypes? These questions are addressed in chapter III.

How do these mitochondrial mutations extend lifespan? Because mitochondria are the major source of ROS, which could potentially accelerate aging, a simple explanation for the increased longevity of animals with reduced respiration is that they generate less ROS as the animal ages. However, timed RNAi experiments indicate that respiratory-chain activity must be reduced during development for lifespan extension. Adult-only RNAi treatments reduce ATP levels and slow behavioral rates but do not extend lifespan (Dillin, Hsu et al. 2002; Rea, Ventura et al. 2007). If reducing mitochondrial respiration extended lifespan by reducing the level of ROS produced during the aging process itself, then one might expect reducing respiration at any time, not only in development, would extend

lifespan (Dillin, Hsu et al. 2002). In addition, lifespan extension does not correlate with resistance to oxidative stressors (Lee, Lee et al. 2003;Rea, Ventura et al. 2007).

clk-1 mutations, on the other hand, have very little effect on respiration, hence it is unlikely they are reducing ROS in this way. Overexpression of *clk-1* shortens lifespan and increases movement rates in *C. elegans* (Felkai, Ewbank et al. 1999) suggesting that whatever the mechanism, its influence on longevity and rates of living is rate-limiting in the animal. However the exact mechanism remains elusive.

In chapter III we present experimental evidence for a likely mechanism by which these mitochondrial mutants have increased longevity and introduce a potential regulatory pathway governing their mutant phenotypes.

1.5 - *C. elegans* as a model organism for studying lifespan regulation – *Why old worms?*

The nematode *C. elegans* as a model organism has pioneered the genetic study of aging (Kenyon, Chang et al. 1993; Duhon and Johnson 1995; Klass 1977). This small nematode has been used in elucidating several different biologic phenomena ranging from apoptosis (Ellis and Horvitz 1986; Hengartner, Ellis et al. 1992), RNA interference (Fire, Xu et al. 1998; Timmons, Court et al. 2001), behavioral neuroscience (Bargmann, Thomas et al. 1990) and, more recently, aging. Its genetic tractability, small size (I mm in length), and transparency of its outer cuticle (which allows visualization of all its 959 cells), have made it an extremely used and useful model organism.

Since the 1960s, when the organism was first chosen as a model genetic organism (Brenner 1974), a number of important genes and genetic pathways were first identified in *C. elegans*. Most importantly, several of

these have been shown to be evolutionarily conserved in higher organisms. Importantly, although the study of the genetic regulation of aging is in its relatively early stages, results to date indicate that it is very likely that most mechanisms regulating longevity in *C. elegans* are evolutionarily conserved.

A relatively short lifespan (the mean lifespan of wild type *C. elegans* is approximately two weeks at 20˚C) and the small size of the animals facilitate population studies of longevity. This, in turn, has enabled swift progress in identifying and characterizing a number of genes involved in lifespan regulation.

Some of these genes have proved to be components of known regulatory pathways (such as the IIS pathway, see section 1.4.2), while the role of others is yet to be clarified. In addition, recent technological advances, such as the fully sequenced *C. elegans* genome (1998) and the RNA interference technique (RNAi) (Fire, Xu et al. 1998; Timmons, Court et al. 2001), are now allowing the rapid identification of additional longevity genes.

It is particularly important to note that *C. elegans*, while it is a representative of the clade Ecdysozoa whose ancestor diverged from that of the vertebrates more than 500 million years ago (Aguinaldo, Turbeville et al. 1997), ages in a fashion similar to higher organisms, including humans. Older worms exhibit reduced locomotion and their physiological functions are slowed down (Johnson, Conley et al. 1988). Moreover, detailed analysis of tissue aging in *C. elegans* has demonstrated that aging nematodes, like humans, exhibit sarcopenia, the progressive loss of muscle mass and muscle function (Herndon, Schmeissner et al. 2002). In addition, other tissues, such as the hypodermis and intestine, also suffer age-related damage (Garigan, Hsu et al. 2002; Herndon, Schmeissner et al. 2002). Moreover, lipofuscin, a pigment found in “age spots” in humans, accumulates in aging worms (Klass 1977). The skin of *C. elegans* becomes

wrinkled and pale as the animal ages. Thus, age-specific tissue deterioration gives the animal a characteristic macro- and microscopic appearance similar to that of aging humans.

Mutations that cause increased longevity slow the rates of tissue-specific aging. For instance, the *age-1* mutation (part of the IIS pathway of lifespan extension), which extends longevity by approximately 30 to 50% and is known to enhance locomotory activity in older animals (Duhon and Johnson 1995), delays the onset of age-related sarcopenia (Herndon, Schmeissner et al. 2002). Thus, mutations that cause animals to live longer than wild type may do so by increasing these animals' health span. Health span can be defined as the amount of time before significant decline of physiological function occurs.

Chapter II
Timing of Action of Mitochondrial Mutations

2.1 – Summary

Mitochondrial deficiencies can only increase longevity if caused during development. In this section, I identified the developmental time before which mitochondrial function must be decreased in order to affect lifespan. I found it to be during the L3/L4 stages of post-embryonic development. This suggests that the "decision" to live long in response to mitochondrial dysfunction occurs late in development, just before the final transition to adulthood.

2.2 – Background

As mentioned in section 1.4.3, mitochondrial knock down using RNAi significantly extends longevity, but only if performed during development. Inhibiting mitochondrial function during adulthood has no effect on lifespan (Dillin, Hsu et al. 2002; Rea, Ventura et al. 2007). This supports the notion that the phenotypes observed in these long-lived mitochondrial mutants are somehow regulated. If the increase in lifespan was simply due to a passive process (such as decreased ROS production) one would expect to see some longevity benefit, if only partial, from an adult-only treatment. Bearing this in mind, I was interested in understanding the nature of this developmental regulatory mechanism.

During post-embryonic development under well-fed conditions *C. elegans* goes through four different larval molts (L1-L4). During each of these stages specific developmental events occur, some of which have

been extensively documented. Namely, with regards to mitochondria there is a several-fold increase in mitochondrial number during the L3 and L4 stages, related to sexual maturation (Tsang and Lemire 2003).

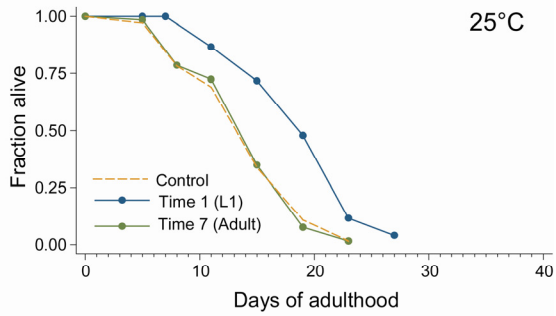
I was interested in finding the important stage for the "decision" regulating longevity in long-lived mitochondrial RNAi-treated animals.

2.3 – Results

In order to identify the "decision" stage for mitochondrial RNAi mutants, I knocked down mitochondrial function at different stages during post-embryonic development and measured longevity of each population (Fig. 4). The knockdowns were achieved using an RNAi clone specific for *cyc-1,* which is a component of complex III of the ETC (Dillin, Hsu et al. 2002). The experiment was done at two different temperatures (25°C and 20°C) with similar results. We found that knocking down mitochondrial function after the L3 stage was insufficient to significantly extend longevity (note that we are using a $p < 0.01$ cut-off for significant differences from control). If we take into account that RNAi by feeding takes ≈ 8-12 hours to decrease RNA levels (Dillin, Hsu et al. 2002; Rea, Ventura et al. 2007), we can estimate the important stage for mitochondrial-mediated longevity to be late L3 or L4. This is consistent with the observation that when animals were exposed to *cyc-1* RNAi from hatching they would often develop at a normal rate and arrest for several hours at the L4 stage (data not shown).

A

	Developmental Stage	Mean Lifespan	*p*-value
Time 1	L1	19.7	$p < 0.001$
Time 2	L1	20.5	$p < 0.001$
Time 3	L2	19.9	$p < 0.001$
Time 4	L3	16.1	$p = 0.12$
Time 5	L3/L4	15.4	$p = 0.56$
Time 6	L4	14.8	$p = 0.87$
Time 7	Adult	14.9	$p = 0.98$
Control	NA	15	NA



B

	Developmental Stage	Mean Lifespan	*p*-value
Time 1	L1	22.7	$p < 0.001$
Time 2	L1	NA	NA
Time 3	L2	25.5	$p < 0.001$
Time 4	L2	24.3	$p < 0.001$
Time 5	L3	22	$p = 0.0053$
Time 6	L3	21	$p = 0.06$
Time 7	L4	20.4	$p = 0.13$
Time 8	L4	19.4	$p = 0.52$
Time 9	Adult	18.3	$p = 0.64$
Control	NA	18.3	NA

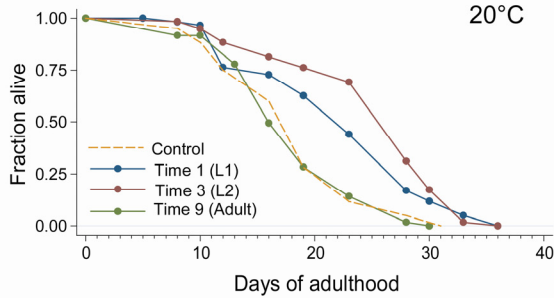


Figure 4 - Mitochondrial knock down after the L3 postembryonic stage is insufficient to increase longevity. Tables show the different developmental stages at which mitochondrial function was impaired and respective lifespans of each population. Greyed lines correspond to non-significant differences relative to control. Note that the significance cut-off when comparing to controls is $p < 0.01$. **A -** Study done at 25°C. Survival graph plots lifespans of populations exposed to *cyc-1* RNAi from hatching (Time 1), only as adults (Time 7) and population exposed to control RNAi (Control). Time 1: N = 70; Time 2: N = 72; Time 3: N = 71; Time 4: N = 71; Time 5: N = 71; Time 6: N = 71; Time 7: N = 71; Control: N = 70. **B -** Study done at 20°C. Survival graph plots lifespans of populations exposed to *cyc-1* RNAi from hatching (Time 1), as L2s (Time 3), only as adults (Time 7) and population exposed to control RNAi (Control). The reason for inclusion of Time 3 was that this population was the longest lived. Time 1: N = 60; Time 2: N = NA; Time 3: N = 62; Time 4: N = 61; Time 5: N = 60; Time 6: N = 60; Time 7: N = 60; Time 8: N = 63; Time 9: N = 60; Control: N = 60.

2.4 – Discussion

Knocking down mitochondrial function during adulthood has no effect in longevity. These experiments show that the decisive stage in the long-life response to mitochondrial dysfunction is L3/L4. This is consistent with previous work showing a big proliferation of mtDNA during this stage of the animals life (Tsang and Lemire 2003), which in turn coincides with reproductive maturation. This energy intensive developmental stage is an optimal candidate for sensing the ability of cells to produce energy, since it is the time where mitochondrial deficiencies would have the most obvious consequences.

Similar findings were published recently (Rea, Ventura et al. 2007), but interestingly where these authors report an “all-or-nothing” effect I saw a gradual decrease in longevity as mitochondrial function was inhibited closer to adulthood. It is interesting to note that when the experiment was done at 20°C, populations in which the RNAi treatment was started as L2s actually lived longer than animals treated from hatching. The reason for this is unclear, however it is possible that starting the treatment too early has detrimental effects. It is also possible that this result was merely experimental variation, since it was not repeated by Rea et. al (Rea, Ventura et al. 2007).

2.5 – Methods

Strains

The strain used in this study was: N2-Bristol (WT)

RNAi feeding at different developmental times

Bacterial feeding RNAi experiments were performed as described previously (Murphy et al. 2003). *cyc-1* RNAi clone was picked from Julie

Ahringer RNAi library and confirmed by sequencing. This RNAi clone was picked based on experiments from Dillin et al., 2002. Populations were synchronized as L1s overnight through larval arrest. Animals were moved from a master population exposed to control RNAi, to plates containing bacteria expressing *cyc-1* dsRNA at different times through development. Timepoints were previously determined to reflect all different stages of post-embryonic development. Each population's developmental stage was determined by visual examination prior to transfer to RNAi treatment.

Survival measurements

Lifespan analysis was conducted as previously described (Dillin, Hsu et al. 2002). Assays were done at 20°C and 25°. The Stata 8.0 software package (Stata Corporation) was used for statistical analysis and to calculate means and percentiles. In all cases p-values were calculated using the logrank (Mantel-Cox) method.

Chapter III

A Regulated Longevity Response to Mitochondrial Respiration in *C. elegans**

***adapted from Cristina, D.**, Cary, M., Lunceford, A., Clarke, C., Kenyon, C., (2008). *A regulated longevity response to mitochondrial dysfunction*. PLoS Genetics. (Submitted)

3.1 - Summary

Mitochondrial respiration generates energy in the form of ATP. When respiration is inhibited in C. elegans, rates of behavior and growth are slowed and lifespan is extended. We find that inhibiting respiration increases the expression of genes predicted to protect and metabolically remodel the animal. This pattern of gene expression is reminiscent of the expression profile of long-lived respiration-defective yeast and mammalian cells, the "retrograde response", suggesting ancient evolutionary conservation. As in yeast, genes switched on in C. elegans mitochondrial mutants extend lifespan, suggesting an underlying evolutionary conservation of mechanism. Mutations in *clk-1*, which inhibit the synthesis of ubiquinone, do not reduce ATP levels, nevertheless they produce gene expression, longevity, and behavioral phenotypes similar to those produced by inhibiting components of the respiratory chain. We find that knocking down the activities of two similar genes, *fsrt-1* and *fstr-2*, partially suppresses the phenotypes of *clk-1* mutants and inhibits the *clk-1(-)* transcriptional response. Thus, *fstr-1/2*, which encode potential signaling proteins, appear to be part of a mechanism that actively slows rates of growth, behavior and aging in response to altered ubiquinone synthesis. Unexpectedly, *fsrt-1/2* are not required for the longevity and behavioral

phenotypes produced by inhibiting the gene *isp-1*, which encodes a component of the respiratory chain. Our findings suggest that different types of mitochondrial perturbations activate distinct pathways that converge on similar downstream processes to slow behavioral rates and extend lifespan.

3.2 - Background

The link between mitochondria and aging is fascinating and complex. Mitochondria generate most of the cell's energy as well as its reactive oxygen species (ROS), and mitochondrial dysfunction can cause disease and accelerate aging. Paradoxically, mitochondrial dysfunction can also increase longevity. Yeast petite mutants, which lack mitochondrial DNA and do not carry out respiration, have an increased replicative lifespan (Kirchman, Kim et al. 1999). In *C. elegans*, two types of mutations that affect mitochondrial function also increase lifespan. The first type reduces respiration. One such mutant, *isp-1(qm150),* was identified in an EMS screen for mutants with delayed development and defecation rates. These animals harbor a mutation in an iron-sulfur protein in complex III of the electron transport chain and have reduced rates of oxygen consumption (Feng, Bussiere et al. 2001). In addition, two independent RNA interference (RNAi) longevity screens found that knock-down of genes encoding components of the respiratory chain or ATP synthase decreased ATP production and rates of respiration, reduced behavioral rates and increased lifespan (Dillin, Hsu et al. 2002; Lee, Lee et al. 2003). Mitochondrial RNAi-treated animals are smaller than *isp-1* mutants (Dillin, Hsu et al. 2002) (Hekimi and Guarente 2003), implying either a more severe reduction in respiration or, conceivably, a qualitatively different response.

The second type of mitochondrial mutant is exemplified by *clk-1* mutants, which are also long lived and have reduced behavioral rates

(Lakowski and Hekimi 1996). *clk-1* mutants lack a mitochondrial hydroxylase necessary for synthesis of ubiquinone, a prenylated benzoquinone lipid required for shuttling electrons from complexes I and II to complex III during respiration (Ewbank, Barnes et al. 1997). In yeast, the *clk-1* homologue *COQ7* is necessary for respiration, and *coq7* mutants are unable to grow on non-fermentable carbon sources. In contrast, *C. elegans clk-1* mutants are not only viable, but they have close to normal levels of respiration and ATP (Braeckman, Houthoofd et al. 1999; Felkai, Ewbank et al. 1999). *clk-1* mutants compensate for the lack of endogenous ubiquinone, Q_9 (the subscript refers to the number of isoprene units) with bacterial Q_8, provided in their diet (Jonassen, Larsen et al. 2001; Larsen and Clarke 2002). In the absence of *clk-1*, the animals accumulate the Q_9 precursor demethoxyubiquinone (DMQ_9). There is some debate over what role DMQ_9 plays in the *clk-1* phenotypes, but the data suggest that they are likely due to absence of Q_9 (Larsen and Clarke 2002; Branicky, Nguyen et al. 2006; Padilla, Jonassen et al. 2004). A role for *clk-1* in lifespan determination may be conserved evolutionarily, since mice with reduced levels of *Mclk-1* are also long lived (Liu, Jiang et al. 2005).

How do these mitochondrial mutations extend lifespan? Because respiration is the major source of ROS, which could potentially accelerate aging, a simple explanation for the increased longevity of animals with reduced respiration is that they generate less ROS as the animal ages. However, timed RNAi experiments indicate that respiratory-chain activity must be reduced during development for lifespan extension. Adult-only RNAi treatments reduce ATP levels and slow behavioral rates but do not extend lifespan (Dillin, Hsu et al. 2002; Rea, Ventura et al. 2007). If reducing mitochondrial respiration extended lifespan by reducing the level of ROS produced during the aging process itself, then one might expect reducing respiration at any time would extend lifespan. In addition, lifespan extension does not correlate with resistance to the oxidative stressor

paraquat (Lee, Lee et al. 2003). Likewise, little is known about the mechanism by which *clk-1* mutations, which have very small effects on respiration, extend lifespan. Overexpression of *clk-1* shortens lifespan and increases movement rates in *C. elegans* (Felkai, Ewbank et al. 1999) suggesting that whatever the mechanism, its influence on longevity and rates of living is rate-limiting in the animal.

In this study, we have asked how mitochondrial mutations affecting respiration and ubiquinone biosynthesis might extend the lifespan of *C. elegans*. It seems possible that these mutations extend lifespan in a similar way, since their effects on growth and behavioral rates are so similar. In yeast, loss of mitochondrial DNA triggers a robust transcriptional response. This change in gene expression is known as a "retrograde response", so named because it implies a reversal in the normal direction of information flow between the mitochondria and nucleus (Liao and Butow 1993; Kirchman, Kim et al. 1999; Parikh, Morgan et al. 1987). The genes expressed during the retrograde response lead to a metabolic remodeling of the cell, heat-shock resistance, and increased mitochondrial biogenesis (Epstein, Waddle et al. 2001; Traven, Wong et al. 2001; Butow and Avadhani 2004; Miceli and Jazwinski 2005). The retrograde response has been shown to be required for the increased longevity of these so-called yeast "petites". Thus, lifespan extension in these yeast cells is actively regulated, and not simply a passive consequence of decreased respiration. Yeast are not the only cells that exhibit a retrograde response to loss of respiration. A similar response has been observed in cultured mammalian cells when mitochondrial DNA is depleted using ethidium bromide (Miceli and Jazwinski 2005).

The retrograde response may be a compensatory reaction to the normal decline in mitochondrial function seen with age, since it is up-regulated in older cells (Kirchman, Kim et al. 1999). Whether it could potentially play a role in longevity determination in multicellular organisms is not known.

Consistent with this possibility, *C. elegans isp-1* mutants have increased levels of expression of at least one protective gene, the superoxide dismutase *sod-3* (Feng, Bussiere et al. 2001). In this study, we carried out microarray analysis of *C. elegans* mitochondrial mutants to better understand the molecular basis of their longevity.

3.3 - Results

3.3.1 - *clk-1* mutants exhibit a conserved retrograde response

clk-1 mutations are particularly interesting because their phenotype is so enigmatic and their effects on longevity appear to be evolutionarily conserved (Liu, Jiang et al. 2005). For gene expression profiling, we grew synchronized populations of *clk-1(qm30)* mutants and wild-type (N2) animals and collected them as pre-fertile adults. 1007 genes were deemed significant and ranked using the SAM (Significance Analysis of Microarrays) tool (Tusher, Tibshirani et al. 2001) using a False Discovery Rate (FDR) of ~0.1 as cut-off. Interestingly, the majority of the genes in this group (99%) were up-regulated relative to wild type, as was also the case in yeast petites (Epstein, Waddle et al. 2001; Traven, Wong et al. 2001). The genes were analyzed for significant overrepresentation of Gene-Ontology (GO) categories using the software BiNGO (Maere, Heymans et al. 2005). Several GO categories were overrepresented in *clk-1* mutants (Table 1) and were related to different cellular processes. From the overrepresented GO categories, it appears that *clk-1* mutants undergo significant metabolic reorganization and activate a stress response similar to that elicited by xenobiotics. For example, GO categories 6006, 6007, and 6096 include genes involved in glycolysis; GO categories 16835 and 44275 include genes potentially involved in glycolysis, gluconeogenesis or anaplerotic pathways; GO category 32787 contains genes involved in anaplerotic reactions (which generate Krebs cycle intermediates); GO categories 9072,

9074 and 30170 encompass genes involved in amino acid metabolism; GO categories 6825 and 5375 include genes involved in Cu transport; GO category 6629 includes genes involved in lipid metabolism; GO category 46040 includes genes involved in nucleotide metabolism; and GO categories 4499 and 16758 include genes involved in xenobiotic response and maintenance of cellular redox state.

When we looked at the level of individual genes, we observed up-regulation of genes encoding enzymes required for glycolysis, such as GPD-2, GPD-3, (gliceraldehyde 3-phosphate dehydrogenase), T05D4.1 (Aldolase A homologue), and LDH-1 (lactate dehydrogenase). GEI-7, which is an enzyme necessary for the glyoxylate cycle in worms (see section 3.4.1), was also up-regulated. We also observed increased expression of an isocitrate dehydrogenase C30G12.2, likely involved in the Krebs cycle, and other alcohol dehydrogenases (*dhs-29*, *dhs-3*) that could potentially act in anaplerotic pathways. We found increased expression of proteins involved in oxidative phosphorylation such as *asg-2* (subunit of ATP synthase complex) and F17A9.4 (NADH oxidoreductase). Also, several genes coding for enzymes involved in amino acid and nucleotide metabolism showed increased expression in *clk-1* mutants. There was also a significant increase in enzymes involved in cellular detoxification, including UDP-glycosyl transferases (UGT-53, UGT-13, UGT-43, UGT-6, UGT-39), gluthathione S-transferases (GST-4, GST-13, GST-36), superoxide dismutase (SOD-3), flavin-containing monooxygenases (FMO-1, FMO-3) and other gene classes potentially involved in xenobiotic metabolism (cytochrome P450s, alcohol dehydrogenases and ABC transporters).

To compare the retrograde response of *clk-1* mutants to the yeast retrograde response, we referred to two previous publications (Epstein, Waddle et al. 2001; Traven, Wong et al. 2001) studying the effects of inhibiting mitochondrial respiration in yeast petites. We applied BiNGO

software to the most differentially expressed genes reported in those studies and compared the results to those of *clk-1* mutants. Among the top ranking GO categories ($p < 0.1$), we observed a remarkable degree of similarity ($p < 0.001$) between the *clk-1* and yeast petite BiNGO results (Table 1).

One of the hallmarks of the yeast retrograde response is an increase in mitochondrial biogenesis (Biswas, Adebanjo et al. 1999). To test whether there was an increase in mitochondrial biogenesis in *clk-1* mutants, we used Real-Time qPCR to quantify mitochondrial DNA. We observed a significant increase in mitochondrial DNA levels (Fig. 5). Together these data suggest that *clk-1* mutants express a transcriptional response to mitochondrial dysfunction that is similar to the yeast retrograde response.

A

	GO ID	Category	*clk-1(-)*	*cyc-1(RNAi)*	*isp-1(-)*	Yeast "petites"
Biological Process	8152	metabolic process	6.53E-04	2.05E-02	4.60E-04	3.90E-07
	9058	-biosynthetic process	NS	4.12E-02	3.43E-03	3.39E-13
	44249	-cellular biosynthetic process	2.35E-02	3.02E-02	5.14E-04	2.69E-04
	51186	-cofactor metabolic process	1.08E-02	NS	1.80E-04	1.66E-09
	6733	-oxidoreduction coenzyme metabolic process	4.42E-02	NS	1.75E-03	1.84E-04
	51188	-cofactor biosynthetic process	1.58E-03	NS	3.83E-03	7.82E-03
	9108	-coenzyme biosynthetic process	2.18E-02	NS	3.28E-04	3.23E-02
	6066	-alcohol metabolic process	1.15E-04	NS	8.32E-02	2.54E-04
	46164	-alcohol catabolic process	2.98E-04	NS	7.01E-02	2.03E-02
	5975	-carbohydrate metabolic process	2.85E-03	NS	3.26E-02	6.95E-09
	44262	-cellular carbohydrate metabolic process	3.10E-04	NS	5.79E-02	4.34E-07
	5996	-monosaccharide metabolic process	2.32E-04	7.16E-02	1.29E-02	1.68E-03
	46365	-monosaccharide catabolic process	2.17E-04	NS	5.99E-02	1.52E-02
	19318	-hexose metabolic process	2.05E-04	6.72E-02	1.18E-02	3.36E-03
	19320	-hexose catabolic process	2.17E-04	NS	5.99E-02	3.90E-02
	6006	-glucose metabolic process	4.23E-05	NS	2.01E-02	4.59E-04
	6007	-glucose catabolic process	2.17E-04	NS	5.99E-02	2.92E-02
	6096	-glycolysis	7.11E-05	NS	3.41E-02	7.90E-03
	6725	-aromatic compound metabolic process	4.65E-04	3.73E-02	NS	NS
	9072	-aromatic amino acid family metabolic process	1.63E-03	8.30E-03	NA	NS
	41	transition metal ion transport	3.67E-03	1.79E-02	1.33E-02	6.84E-02
	6825	-copper ion transport	1.63E-03	6.84E-02	6.13E-03	NS
	7568	aging	NS	9.86E-03	1.04E-06	NA
	10259	-multicellular organismal aging	NS	9.86E-03	1.04E-06	NA
	8340	-determination of adult life span	NS	9.86E-03	1.04E-06	NA
Molecular Function	3674	molecular_function	2.86E-03	4.50E-05	NS	NS
	48037	-cofactor binding	3.36E-05	2.13E-02	NS	NA
	50662	-coenzyme binding	1.23E-03	9.50E-02	NS	NA
	3824	-catalytic activity	1.48E-06	3.57E-02	NS	NS
	16491	-oxidoreductase activity	2.62E-08	3.10E-03	6.19E-02	2.53E-05
	16709	-oxidoreductase activity, NADH or NADPH as one donor, and incorporation of one atom of oxygen	6.94E-03	1.18E-03	NA	NA
	4499	-flavin-containing monooxygenase activity	5.02E-03	6.91E-04	NA	NA
	16757	-transferase activity, transferring glycosyl groups	9.59E-04	4.96E-03	NS	NA
	16758	-transferase activity, transferring hexosyl groups	1.65E-03	5.69E-03	NS	NS
	16835	-carbon-oxygen lyase activity	6.35E-02	5.61E-03	4.09E-04	3.90E-02
	15082	-di-, tri-valent inorganic cation transmembrane transporter activity	4.34E-03	2.09E-02	NS	NS
	46915	-transition metal ion transmembrane transporter activity	1.63E-03	NS	5.64E-02	9.78E-02
	5375	-copper ion transmembrane transporter activity	9.67E-04	NS	4.13E-02	NA
	5198	-structural molecule activity	1.55E-02	2.32E-04	NS	6.94E-20
Cellular Component	44424	intracellular part	NS	8.67E-02	4.21E-03	3.68E-03
	267	cell fraction	9.67E-04	5.03E-03	NA	NS
	5624	-membrane fraction	9.67E-04	5.03E-03	NA	NS
	42598	-vesicular fraction	5.06E-04	2.69E-03	NA	NA
	5792	-microsome	5.06E-04	2.69E-03	NA	NA

B

	GO ID	Category	*clk-1(-)*	*cyc-1(RNAi)*	*isp-1(-)*	Yeast "petites"
Biological Process	3	reproduction	NS	NS	3.07E-04	NA
	6629	lipid metabolic process	3.74E-03	NS	NS	NS
	6820	anion transport	NS	3.08E-05	NS	2.97E-03
	15698	inorganic anion transport	NS	1.76E-05	NS	1.96E-02
	6817	phosphate transport	NS	6.35E-05	NS	5.05E-02
	16052	carbohydrate catabolic process	1.98E-03	NS	NS	5.86E-02
	44275	cellular carbohydrate catabolic process	1.58E-03	NS	NS	5.86E-02
	32501	multicellular organismal process	NS	NS	1.17E-10	NA
	32502	developmental process	NS	NS	3.72E-12	NS
	7275	multicellular organismal development	NS	NS	2.96E-12	NA
	9790	embryonic development	NS	NS	1.50E-10	NA
	9792	hatching	NS	NS	6.26E-11	NA
	48513	organ development	NS	NS	4.12E-03	NA
	40008	regulation of growth	NS	NS	8.17E-04	NA
	40009	regulation of growth rate	NS	NS	3.10E-04	NA
	45927	positive regulation of growth	NS	NS	3.51E-04	NA
	40010	positive regulation of growth rate	NS	NS	3.05E-04	NA
	44237	cellular metabolic process	NS	NS	1.53E-03	2.44E-03
	6082	organic acid metabolic process	3.12E-04	NS	NS	8.35E-06
	19752	carboxylic acid metabolic process	3.12E-04	NS	NS	8.35E-06
	32787	monocarboxylic acid metabolic process	1.99E-03	NS	NS	2.00E-06
	6139	metabolic process	NS	NS	1.72E-05	NS
	6188	IMP biosynthetic process	3.39E-03	NA	NA	NA
	6259	DNA metabolic process	NA	NA	7.44E-04	NS
	19363	pyridine nucleotide biosynthetic process	3.39E-03	NA	NS	NS
	46040	IMP metabolic process	3.39E-03	NA	NA	NA
	6732	coenzyme metabolic process	NS	NS	6.82E-05	8.49E-08
	19439	aromatic compound catabolic process	1.04E-03	NA	NA	NA
	9074	aromatic amino acid family catabolic process	1.04E-03	NA	NA	NA
	42375	quinone cofactor metabolic process	NA	NS	1.34E-03	4.14E-02
	6743	ubiquinone metabolic process	NA	NS	1.34E-03	4.14E-02
	6744	ubiquinone biosynthetic process	NA	NS	1.34E-03	4.14E-02
	45426	quinone cofactor biosynthetic process	NA	NS	1.34E-03	4.14E-02
	48518	positive regulation of biological process	NS	NS	1.33E-04	NS
Molecular Function	3868	4-hydroxyphenylpyruvate dioxygenase activity	NA	1.12E-03	NA	NA
	4497	monooxygenase activity	NS	3.78E-05	NA	NA
	5506	iron ion binding	NS	7.77E-04	NS	NA
	8138	activity	NA	NS	6.29E-04	NA
	16836	hydro-lyase activity	NS	NS	1.39E-03	1.44E-02
	30170	pyridoxal phosphate binding	4.92E-03	NS	NS	NA
	33170	DNA-protein loading ATPase activity	NA	NA	2.64E-03	NA
	3689	DNA clamp loader activity	NA	NA	2.64E-03	NA
	46872	metal ion binding	NS	8.64E-04	NS	6.32E-02
Cellular Component	5739	mitochondrion	NS	NS	3.83E-03	2.81E-46
	30054	cell junction	1.74E-03	NS	NS	NA
	5911	intercellular junction	1.52E-03	NS	NS	NA
	5921	gap junction	1.13E-03	NS	NS	NA
	14704	intercalated disc	1.13E-03	NS	NS	NA
	32991	macromolecular complex	NS	NS	1.01E-03	2.94E-02
	5663	DNA replication factor C complex	NA	NA	2.64E-03	NA
	42302	structural constituent of cuticle	NS	6.80E-06	NS	NA
	44428	nuclear part	NA	NS	6.93E-04	NS

Table 1 - Up-regulated GO categories in worm and yeast mitochondrial mutants. Tables 1A and 1B show the p-values for the most significant GO categories found in *isp-1(-)*, *clk-1(-)* and *cyc-1(RNAi)* animals ($p < 0.1$; FDR < 0.1 in at least one mutant) (red represents more significant, yellow represents less significant). The "yeast petite" column indicates whether each GO category found in one or more of the *C. elegans* mutants was also overrepresented in petite yeast data sets. GO categories are aggregated into GO "branches" (same cell in table) if they are part of the same GO hierarchy and are directly related to each other. NS represents non-significant, for GO categories that were below our cut-off. NA represents not available, for GO categories that had no annotated genes in yeast or for situations in which no expression data was available for that category. GO categories were separated into two tables (A and B). **A.** GO categories present in two or more *C. elegans* mitochondrial mutants. All overlap sets, both between different combinations of two mutants, as well as between all three mutants, are highly significant ($p < 0.001$). In addition, the number of categories shared between all three *C. elegans* mutants and yeast "petites" is also much higher than expected by random ($p < 0.001$; one would expect 2 categories by random chance) **B.** GO categories present in only one mitochondrial mutant. Categories that were present in only one mutant were also less likely to be present in the yeast petite dataset. Note that cell-protective genes are not well annotated as distinct GO classes in current *C. elegans* data bases (ie, WormBase).

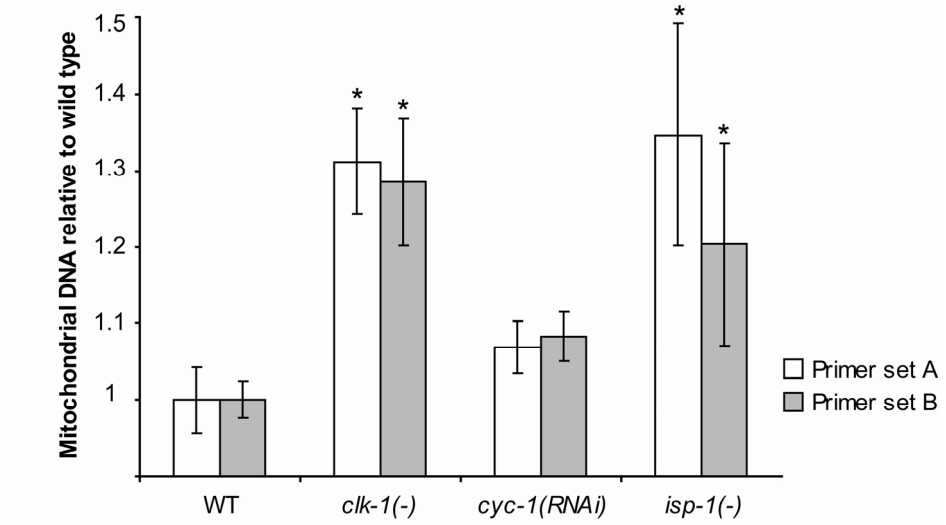


Figure 5 - Mitochondrial DNA quantification. Percent increase in total mitochondrial DNA relative to wild type, as measured by qPCR. Data are shown for two different mitochondrial primer pairs across five independent biological repeats. (*) indicates significance of $p < 0.05$ after Bonferroni multiple comparison correction. Error bars are ± SEM. *clk-1(-)* primer set A: m (mean) = 1.31 ± 0.07, primer set B: m = 1.29 ± 0.08; *cyc-1(RNAi)* primer set A: m = 1.07 ± 0.03, primer set B: m = 1.08 ± 0.03; *isp-1(-)* primer set A: m = 1.35 ± 0.14, primer set B: m = 1.21 ± 0.13.

3.3.2 - *fstr-1/2* and *aqp-1* contribute to the increased longevity of *clk-1* mutants

To identify genes necessary for the increased longevity of *clk-1(-)* animals, we compiled a list of differentially expressed genes from populations of L4 (late larval stage) as well as prefertile adults (see Methods). We included L4 animals in this set because other experiments have shown that the L4 period is the critical period for lifespan determination in at least some mitochondrial mutants (Rea, Ventura et al. 2007). We picked the 75 top differentially expressed genes ranked using the SAM software package, inhibited their functions using RNAi and

measured lifespan. Out of the initial list of 75, we collected lifespan data for 63 genes over two independent trials. We established a significance cut-off of $p < 0.05$ and selected RNAi clones that decreased *clk-1* longevity significantly in both trials or were statistically significant in one experiment and showed a decrease of at least 5% in the other (a 5% decrease in overall lifespan corresponds to an ~25% decrease in the lifespan extension produced by *clk-1* mutation). We retested the positives in *clk-1(-)* and wild-type animals for effects on longevity. Out of 63 RNAi clones tested, only two decreased *clk-1* mutant longevity in all three trials (Fig. 6). Neither of these clones significantly shortened wild-type lifespan, suggesting that they may play a role specifically in *clk-1* mutant lifespan. One of these clones corresponded to *aqp-1,* which encodes a glycerol channel (Huang, Lamitina et al. 2007). *aqp-1* RNAi decreased the lifespan extension that would normally be produced by *clk-1* mutations from 26% to 7%, and did not affect wild-type longevity in two separate experiments. Interestingly, *aqp-1* (also called *dod-4*) has already been shown to contribute to the long lifespan of *daf-2* insulin/IGF-1-receptor mutants (Murphy et al. 2003). The other RNAi clone, corresponding to a gene we call *fstr-1* (for “faster”, also known as *gfi-1*) decreased the lifespan extension produced by *clk-1* mutations from 26% to 3% (close to total suppression on this trial) while not affecting wild-type lifespan in two separate experiments. The effects of *aqp-1* RNAi and *fstr-1* RNAi on the longevity of *clk-1* mutants were tested three times, with consistent results, although the extent of suppression varied between experiments. We examined the genome for the possibility that *fstr-1* RNAi might cross-inhibit another gene, and found that the RNAi clone was likely to knock down a close homolog (with 98% inferred DNA sequence similarity) located next to *fstr-1* that did not exhibit *clk-1*-dependent regulation in our microarray analysis. We call this gene *fstr-2,* and henceforth we refer to their combined functions, as inferred from RNAi, as *fstr-1/2* function.

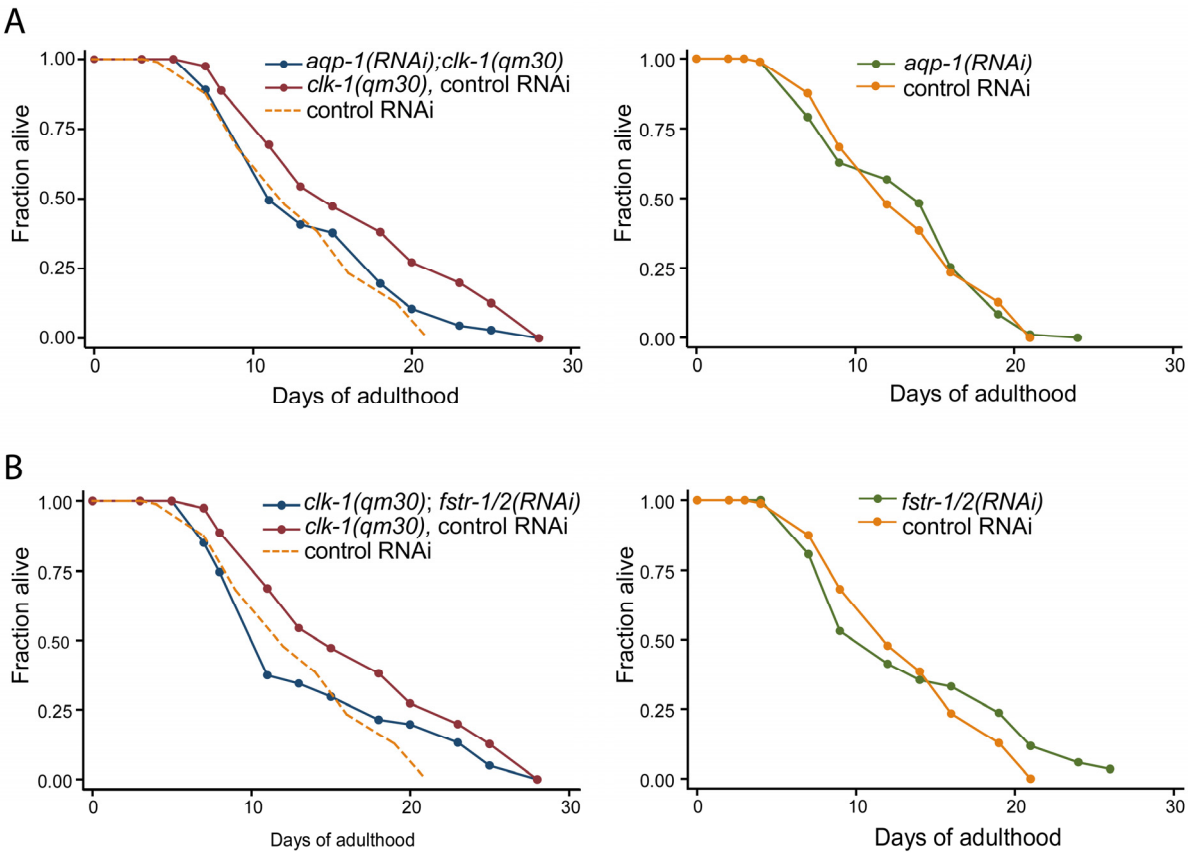


Figure 6 - *fstr-1/2* and *aqp-1* contribute to the increased longevity of *clk-1* mutants A. *aqp-1* RNAi significantly decreased the lifespan extension produced by *clk-1* mutations from 26% (control) to 7%; $p < 0.05$, and had no significant effect on wild type. WT subjected to control (vector-only) RNAi: N = 103, m = 13.4 days. WT subjected to *aqp-1* RNAi: N = 106, m = 13.4 days; *clk-1(-)* subjected to control RNAi: N = 105, m = 16.9 days; *clk-1(-)* subjected to *aqp-1* RNAi: N = 105, m = 14.4 days. Two subsequent independent trials showed a significant decrease in the lifespan extension produced by *clk-1* mutations (from a 17% to 0% increase and from a 33% to 20% increase. **B**. *fstr-1/2* RNAi significantly decreased the lifespan extension produced by *clk-1* mutation from 26% to 3%, $p < 0.05$, while having no significant effect on wild-type longevity. WT subjected to control RNAi, same as in Fig. 2A; WT subjected to *fstr-1/2* RNAi: N = 105, m = 13.9 days; *clk-1(-)* subjected to control RNAi: same as in fig.2A; *clk-1(-)* subjected to *fstr-1/2* RNAi: N = 108, m =

13.8 days. In two other trials, *fstr-1/2* RNAi reduced the lifespan increase produced by *clk-1* mutations from 33% to 27% (not statistically significant) and from 17% to 0%.

3.3.3 - *fstr-1/2* knockdown increases the behavioral rates of *clk-1* mutants

In addition to increased longevity, the most striking phenotypes of mitochondrial mutants are their decreased behavioral rates. In principle, these rates could decrease as a direct consequence of impaired mitochondrial function. Alternatively, it is possible that their slowed behavioral rates reflect a regulated response to mitochondrial perturbation; for example, to conserve energy. To look for genes that slow the behaviors of *clk-1* mutants, we inhibited the top 100 up-regulated genes from microarrays of L4 and pre-fertile adults using RNAi and measured time it took for L1 larvae to develop to adulthood. We found that knockdown of *fstr-1/2* in *clk-1* mutants consistently increased the rate of growth to adulthood (Fig. 7A). This phenotype was most striking when the animals were examined 75-80 hours after hatching. At this time, no control animals had reached adulthood, whereas 95-100% of the *fstr-1/2* RNAi treated animals were adults. *fstr-1/2* RNAi treatment also increased the behavioral rates of *clk-1(-)* animals, as measured by thrashing and pumping (Fig 7B and 7C). These effects were not observed in wild type; in fact, in wild type, knock-down of these genes had the opposite effect, slowing development and decreasing rates of thrashing and pumping.

Until now, only a few *clk-1* suppressors have been found, and these were later found to be informational tRNA missense suppressors that restored *clk-1* function (Branicky, Nguyen et al. 2006). To test whether *fstr-1/2* RNAi somehow restored wild-type *clk-1* function, we examined ubiquinone profiles. Using HPLC, we observed the expected decrease in UQ_9 and increase in DMQ_9 in *clk-1* mutants, and we observed the same

mutant pattern of ubiquinone species in *clk-1* mutants subjected to *fstr-1/2* RNAi (Fig. 7D). Thus, *fstr-1/2* RNAi suppresses the phenotypes of animals that have an altered, *clk-1(-)* pattern of ubiquinone species. This suggests that the wild-type *fstr-1/2* gene helps to slow behavior and extend lifespan in response to the changes in ubiquinone produced by *clk-1* mutations.

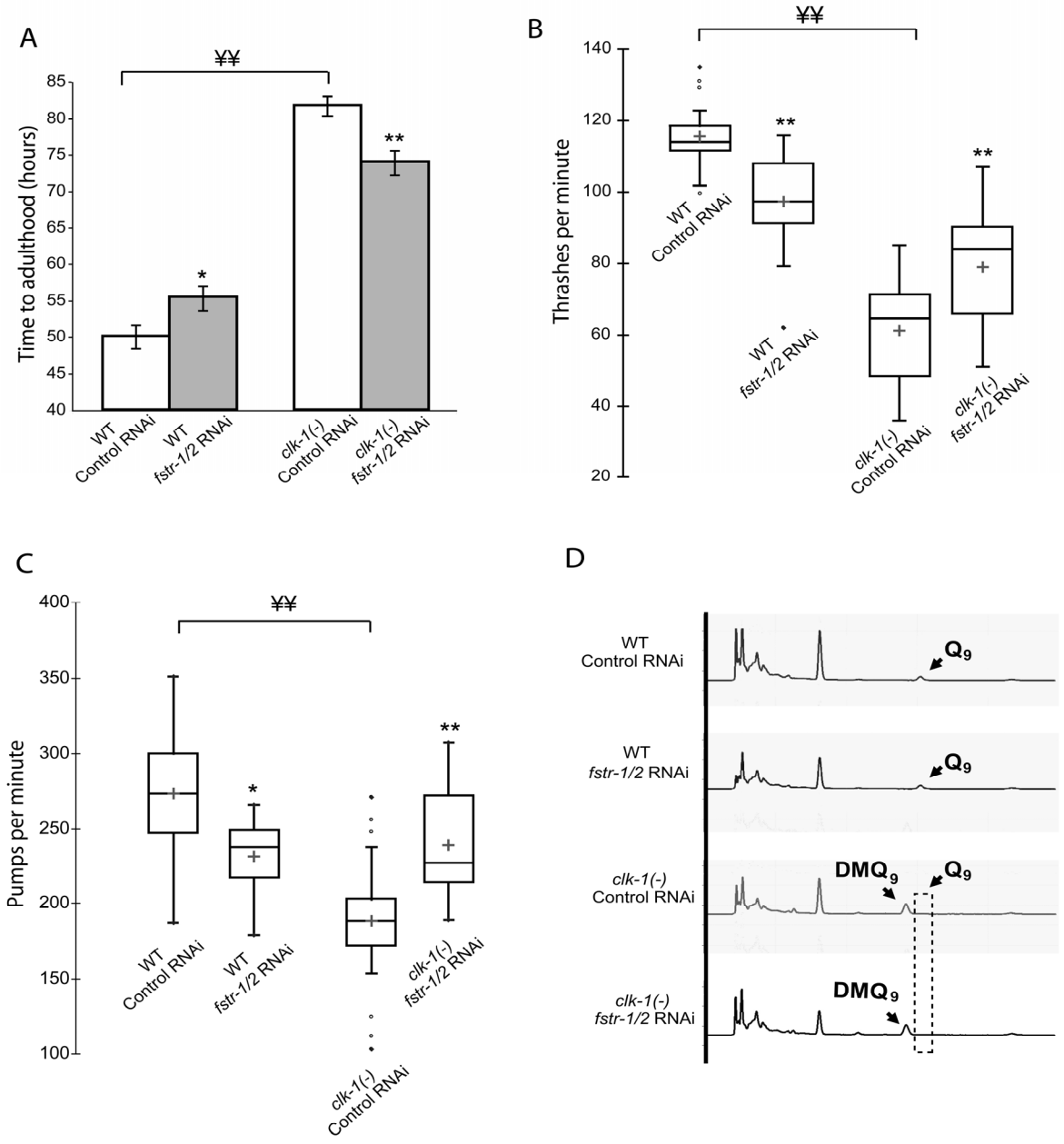


Figure 7 - *fstr-1/2* RNAi speeds up *clk-1(-)* animals. A. Time to adulthood. *clk-1* mutants take much longer to reach adulthood than wild-type animals. This rate of development is accelerated by *fstr-1/2* RNAi. In contrast, *fstr-1/2* RNAi slowed the growth rate of wild type. These effects were very robust and were observed in

every experiment. WT subjected to control RNAi: 47.4 ± 1.6 hours; WT subjected to *fstr-1/2* RNAi: 53.2 ± 1.7 hours; *clk-1(-)* mutants subjected to control RNAi: 82.2 ± 1.2 hours; *clk-1(-)* mutants subjected to *fstr-1/2* RNAi: 72 ± 1.7 hours. **B**. Boxplots illustrating thrashing rates measured on day 3 of adulthood. *fstr-1/2* RNAi treatment significantly reduced the average thrashing rate of wild-type animals and significantly increased the average thrashing rate of *clk-1(-)* mutants. WT subjected to control RNAi: 115.6 ± 3.1 thrashes/min; WT subjected to *fstr-1/2* RNAi: 97.5 ± 4.3 thrashes/min; *clk-1(-)* mutants subjected to control RNAi: 61.2 ± 5 thrashes/min; *clk-1(-)* mutants subjected to *fstr-1/2* RNAi: 78.9 ± 5.7 thrashes/min. **C**. Boxplots illustrating pumping rates. *fstr-1/2* RNAi decreased the average pumping rate of wild type and increased the pumping rate of *clk-1(-)* mutants. WT subjected to control RNAi: 273.3 ± 41.1 pumps/min; WT subjected to *fstr-1/2* RNAi: 231.8 ± 24.7 pumps/min; *clk-1(-)* mutants subjected to control RNAi: 188.5 ± 41 pumps/min; *clk-1(-)* mutants subjected to *fstr-1/2* RNAi: 238.9 ± 36.4 pumps/min. In figures 3A, 3B and 3C error bars depict SEM; * depicts a significance of $p < 0.05$ when compared to controls, ** depicts significance of $p < 0.008$ when compared to controls, ¥¥ depicts significance of $p < 0.008$. $P < 0.008$ is the cut-off set by the Bonferroni correction for multiple comparisons. **D.** HPLC analysis of quinone content. The chromatograms show a representative run of three independent experiments for each of the different conditions (WT subjected to control RNAi, WT subjected to *fstr-1/2* RNAi, *clk-1(-)* mutants subjected to control RNAi and *clk-1(-)* mutants subjected to *fstr-1/2* RNAi). The Q_9 peak is absent from *clk-1*(-) animals and instead the intermediate DMQ_9 is detected. *fstr-1/2* RNAi had no effect in Q_9 levels in wild-type or *clk-1(-)* mutants, in *clk-1* mutants Q_9 levels remained below detection threshold.

3.3.4 - *fstr-1/2* is necessary for gene expression changes observed in *clk-1* mutants

Next we asked whether FSTR-1/2 modulates the *clk-1* mutant phenotype by influencing the retrograde response. Using real-time qPCR, we looked at the effects of *fstr-1/2* RNAi on the expression levels of five of the genes whose expression was most significantly up-regulated genes in

the microarrays: *gpd-2*, a glyceraldehyde 3-phosphate dehydrogenase involved in glycolysis; T22B7.7, an Acyl-CoA thioesterase, involved in anaplerotic reactions; *dhs-26*, an alcohol dehydrogenase; *ugt-43*, an UDP-glucoronosyl transferase; and the aquaporin gene *aqp-1*. The qPCR data confirmed the microarray studies, in that all of these genes were up-regulated in *clk-1* mutants. We found that *fstr-1/2* RNAi significantly and consistently decreased expression of these genes in a *clk-1*(-) background (Fig. 8A) but not in wild type (Fig. 8B). Thus, the gene expression changes observed in *clk-1* mutants are at least partially dependent on *fstr-1/2*. Together these findings suggest that *fstr-1/2(+)* controls rates of behavior and lifespan in *clk-1* mutants by triggering downstream changes in gene expression.

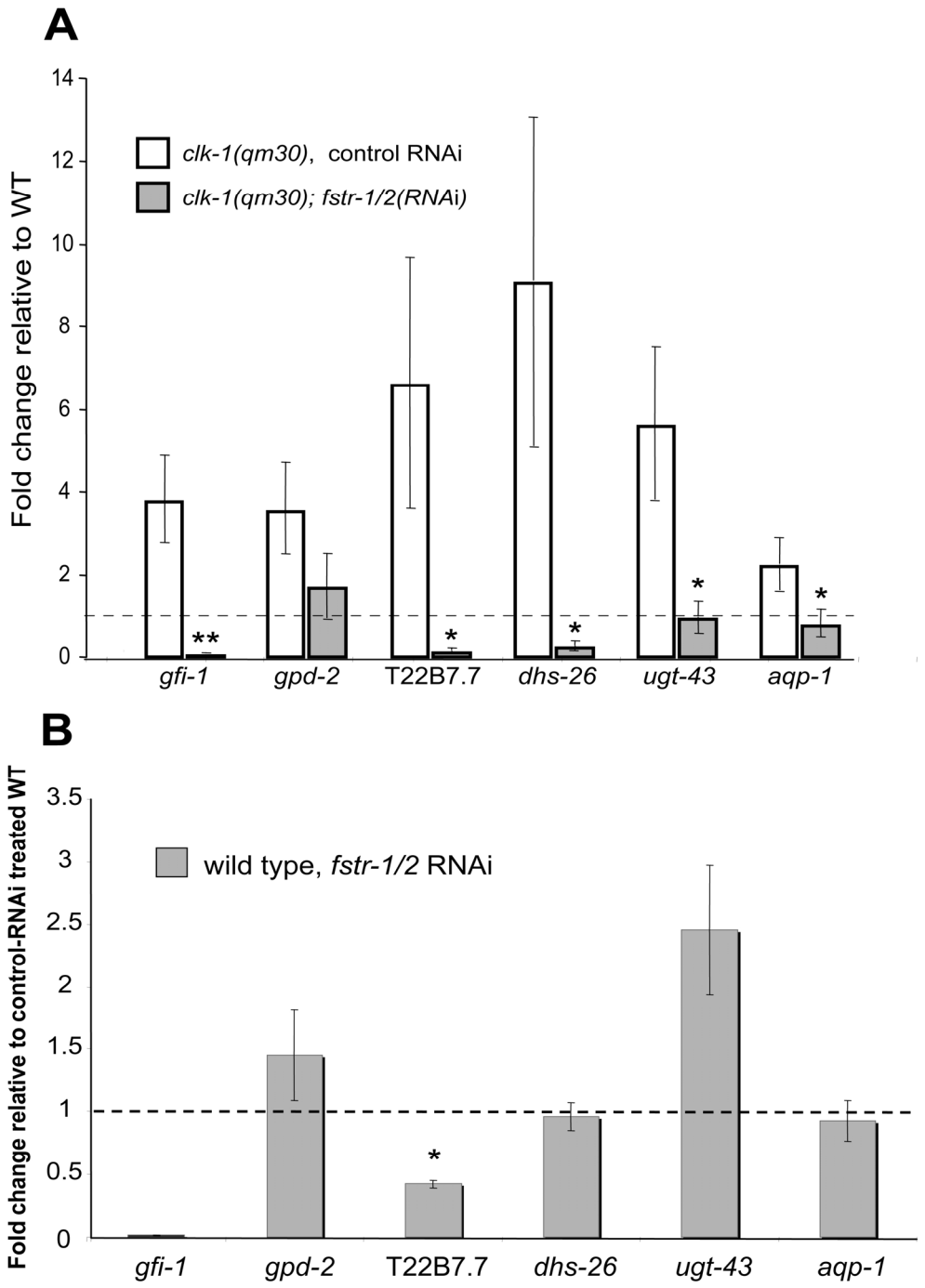


Figure 8 - *fstr-1/2* RNAi inhibits expression of the *clk-1* retrograde response.

A - The graph shows the effect of *fstr-1/2* RNAi on the degree of up-regulation of

individual *clk-1* retrograde-response genes, as determined by quantitative RT-PCR. (**) represents a significance of $p < 0.01$, (*) represents a significance of $p < 0.05$, in comparing mRNA levels from *clk-1(-); fstr-1/2(RNAi)* animals vs. *clk-1(-)* control animals. Each bar represents the average of 3 independent biological repeats. Error bars are ± SEM. *clk-1(-)* animals subjected to control RNAi (grey bars) showed significantly higher expression than did wild type subjected to control RNAi ($p < 0.05$) for all genes tested. With the exception of *gpd-2* all genes showed a significant decrease in expression ($p < 0.05$) in the presence of *fstr-1/2* RNAi. Note that *gpd-2* shared the trend, with p = 0.061, and was clearly affected when assayed using the P*gpd-2::gfp* reporter *in vivo* (Fig. 12) **B -** Retrograde-response genes respond differently to *fstr-1/2* RNAi in wild-type animals and *clk-1* mutants. The graph shows the effect of *fstr-1/2* RNAi on the degree of up-regulation of individual *clk-1* retrograde-response genes, as determined by quantitative RT-PCR in a wild type background. (*) represents a significance of $p < 0.05$, in comparing mRNA levels from wild type animals subjected to *fstr-1/2(RNAi)* vs. wild type control animals. Each bar represents the average of 3 independent biological repeats. Error bars are ± SEM. With the exception of T22B7.7, *fstr-1/2(RNAi)* did not knock down expression of any of these genes in a wild type background.

3.3.5 - *fstr-1* may act in the intestine and/or nervous system to slow down *clk-1* mutants

Because *fstr-1* is up-regulated in *clk-1* mutants, we were particularly interested to learn where in the animal it was expressed. To investigate this, we generated transgenic animals expressing the fluorescent protein mCherry under the control of the *fstr-1* promoter. We observed strong expression in three neurons located in the head and throughout the intestine, particularly in the anterior cells (Fig. 9). The three neurons were identified as RIH and I1L/R. RIH neuron is a nerve-ring interneuron of unknown function and I1L/R are pharyngeal interneurons that regulate pharyngeal pumping rates in response to touch and in the absence of bacteria (Avery L, and Thomas J. H., 1997, *C.elegans II*). We saw the same

pattern of expression in *clk-1(-)* and wild-type, but the intensity of expression was increased in the mutant, consistent with our qRT-PCR and microarray data. Together these findings suggest that *fstr-1* acts in the intestine and/or in specific neurons to slow the rates of aging and behavior in *clk-1* mutants.

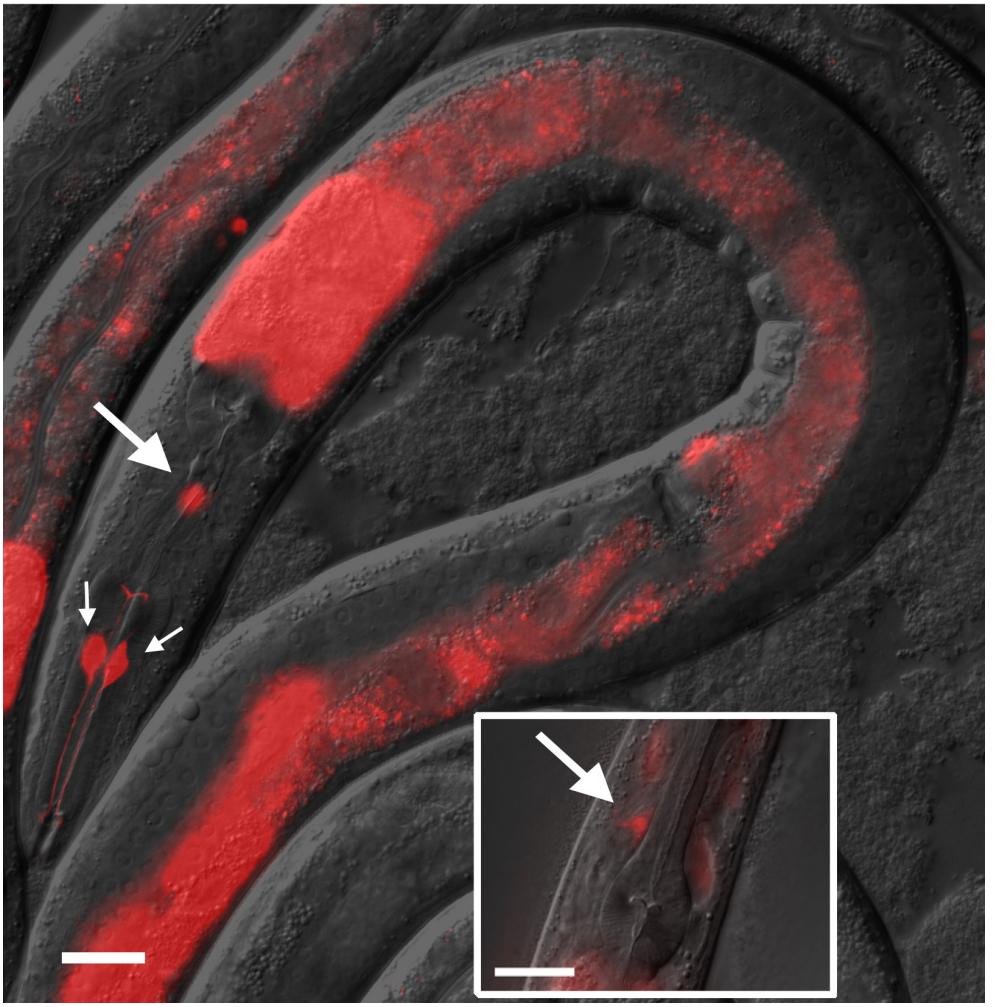


Figure 9 - *fstr-1* is expressed in the intestine and three neurons in a *clk-1(-)* background. A P*fstr-1*::mCherry promoter fusion is expressed in the intestine, especially in anterior cells, as well as in RIH and I1L/R neurons. Small arrows point to cell bodies of I1L/R neurons with their characteristic processes visible; large

arrows point to the cell body of the RIH neuron. The small panel shows a more detailed view of RIH neuron. Scale bar represents 20 µm.

3.3.6 - A similar retrograde response in animals with reduced respiration

To compare the pattern of gene expression in *clk-1* mutants to that of mutants with reduced rates of respiration, we performed microarray analysis of *isp-1(qm150)* and *cyc-1(RNAi)* (cytochrome c) animals. *cyc-1* encodes a cytochrome c reductase which is a component of complex III of the electron transport chain. We grew synchronized populations of *isp-1(qm150)* and *cyc-1(RNAi)* mutants in parallel with wild-type control animals, collected the animals as young, pre-fertile adults and analyzed the resulting microarray data as described above for *clk-1* mutants. The SAM algorithm with a FDR of ~0.1 yielded 814 significant genes for *isp-1(-)* mutants and 7662 significant genes for *cyc-1(RNAi)* animals. Thus, it seems that *cyc-1* RNAi induces a broader transcriptional response than do *clk-1* and *isp-1* mutations, which correlates with the increased severity of the Cyc-1(RNAi) phenotype. In addition, *cyc-1* RNAi-treated animals, when compared to *isp-1* and *clk-1* mutants, showed increased expression of additional cell-protective genes, including chaperones (*hsp-6*, *hsp-70*), superoxide dismutases (*sod-4*, *sod-3*) and xenobiotic detoxification enzymes (*ugt-2*, *ugt-47*, *ugt-36*, *gst-8*, *gst-22*, *gst-24*, *dhs-5*, *dhs-28*). Interestingly, other genes encoding detoxification enzymes were down regulated, possibly implying the deployment of a specific detoxification program. Using BiNGO analysis, we identified several GO categories that were overrepresented in each mutant (Table 1). A highly significant fraction of the top GO categories ($p < 0.1$) were shared between either two, or all three, of the mutant strains ($p < 0.001$; Table 1). By chance, one would expect 2 of the 10 GO categories shared by all three mitochondrial mutants

and annotated in yeast to also be significant in the yeast petite cells; in contrast, we observed 8 categories in common ($p < 0.001$).

In addition to identifying GO categories, we looked for individual genes that were expressed in a similar way in the three *C. elegans* mitochondrial mutants. We found a highly significant ($p = 7.19E-15$) overlap set of 73 differentially-expressed genes (Table 2). In addition, we observed an increase in mitochondrial biogenesis in *isp-1* mutants and a smaller increase in *cyc-1(RNAi)* animals that did not reach statistical significance ($p = 0.354$) (Fig. 5). Taken together, these data suggest that the retrograde responses of different *C. elegans* mitochondrial mutants are similar to one another and to the yeast retrograde response.

		Combined set of arrays (adult + L4)			Adult-only set of arrays		
		% FDR as reported by SAM			% FDR as reported by SAM		
Gene ID	Expression	*clk-1(-)*	*isp-1(-)*	*cyc-1(RNAi)*	*clk-1(-)*	*isp-1(-)*	*cyc-1(RNAi)*
T05D4.1	Up	0.117432495	14.89369246	0	1.829561406	19.28517271	0
Y56A3A.3	Up	0	0.915671504	0	3.108127057	20.80247352	0
R08E5.3	Up	0.206282773	0	0.005583134	1.753472442	12.20068028	0
C54D10.1	Up	0	0.2793282	0.106286148	4.721019493	10.98187461	0
F54D5.7	Up	NA	0	0	21.64044604	47.03401745	15.85357931
DC2.5	Up	0.133900954	5.09257529	0.048563361	1.65080366	39.85812931	3.275615253
F09F7.6	Up	0.20584186	0.339359186	0	3.323660323	2.42683865	NA
F08F3.4	Up	0.025589545	1.265163517	0.058171952	2.778228704	23.08241642	NA
F21C10.10	Up	0.037840306	0.011255965	0	1.539343257	NA	NA
ZK488.2	Up	0.181343276	15.89823588	0.095360376	45.75879869	81.64621888	1.547309504
F37H8.3	Up	26.30310308	51.50273749	0	75.26945402	98.12123468	23.16816374
F58G6.7	Up	0	0	0	4.17014787	15.67249004	6.577453029
K10B3.6	Up	48.8468677	38.86575305	0	33.45627382	92.52922334	0
W05G11.6a	Up	0.18518687	0.137109328	0	1.755783556	11.3857276	7.962261088
C25G4.2	Up	0	43.77214116	0	32.09025598	98.18044421	3.117281717
H25P06.4	Up	0	15.27969306	6.549292917	1.254853431	58.90908725	41.72730549
F52C9.3	Up	11.3422944	7.6344477	0	74.83954329	33.49696819	27.25369083
F54D5.3	Up	20.20245402	7.934356347	0	39.42405085	5.02392454	2.198451317
F32A5.5b	Up	0.071901718	0	0	13.16212079	NA	26.95548603
ZK1320.2	Up	1.716802462	4.833810769	0	48.73248874	70.80757765	35.71508829
C08A9.1	Up	0	0.25544016	0	2.370956896	5.733378742	21.93051454
T28H11.8	Up	17.81832814	62.77854438	0	67.00501663	NA	2.258776319
F58B3.4	Up	76.95978977	4.649983246	15.62956581	82.05366734	28.62512953	29.63209164
F43H9.4	Up	0	16.47085304	0	15.4022445	91.3458612	12.8374886
F53F4.10	Up	0	16.73946668	4.581029505	69.05041964	27.85546302	23.79395877
Y54E5A.8a	Up	2.096019845	28.63945918	5.517252082	52.63826529	88.37272273	55.82994753
F26F12.3a	Up	8.111907217	1.664495572	7.908748974	7.661928709	29.66626736	11.39230145
F40D4.13	Up	1.14085129	0.036961142	0	11.08728282	4.782935813	61.51056523
ZK353.8	Up	29.65864668	14.79062352	31.16164563	62.3430507	19.44626417	NA
F16B4.11	Up	22.8208171	29.36776979	0	53.50491309	48.32187198	NA
K09E3.4	Up	0	10.67875299	12.3251363	1.810757004	69.76683474	31.68053404
T01E8.5	Up	27.17417281	2.595763851	0	63.97652179	2.311810543	55.99145871
E04F6.9	Up	75.56766047	8.67169545	0	47.21837874	43.97871781	6.220131201
K10B3.8	Up	0	0.096949388	0.039117145	1.866181286	20.89763662	52.20052546
K07C10.1	Up	6.584407111	4.712325844	0	32.74002078	NA	36.41313504
C44C10.3	Up	26.84646007	12.471867	0	68.28200123	96.14596198	41.76089255
M05D6.6	Up	16.46034948	6.096348802	14.86886525	59.52513417	19.94708239	10.35507169
C09G12.8a	Up	28.04464047	18.95140585	0	33.69804284	33.50138444	25.15989089
K10B3.7	Up	0	0	3.039630866	6.099766005	NA	NA
Y73B3A.11	Up	0	6.20415241	39.15103374	18.73611744	49.61720447	61.54746505
ZK228.4	Up	0.811584956	2.350180737	0	2.262222384	11.5035587	25.48832582
VW06B3R.1b	Up	21.88821817	18.38431786	13.21619238	21.84235567	32.94655868	8.49129998
K10C8.1	Up	18.09249048	54.69372206	0	45.34320027	96.17729655	56.27286426
F42A10.7	Up	8.81851401	7.736783695	0	24.71215784	NA	26.72378835
C06G3.6	Up	55.05719647	15.15809366	63.08748601	74.49770818	73.96183001	59.82280567
Y71H9A.3	Up	6.927262027	36.44518666	24.85328683	8.033407498	90.69415302	57.50626425
C25G4.3	Up	21.23099168	13.89397923	NA	88.91606458	2.269875152	NA
F43H9.2b	Up	60.58156573	13.47619634	11.29293988	28.40830857	79.34816604	47.9563887
F42A6.9	Up	27.63798769	8.066106567	39.40956881	44.00461905	2.702692923	58.94640448
F44C4.3	Up	0	0.677551467	29.19930162	NA	74.82374	NA
C05D9.8	Up	0	1.308130003	36.67255423	11.92338105	2.469066775	58.50041898
Y37H2A.4	Up	0	2.432339835	48.61303153	4.553678471	NA	60.51808869
Y53F4B.25	Up	49.51967515	27.24590031	NA	82.65573561	82.37600389	NA
F52C9.1a	Up	7.086965098	13.40889187	30.16729209	36.86865347	2.684756733	NA
R144.3	Up	17.35693059	34.82868954	19.11132574	44.39074736	56.35998539	60.92688444
F38E11.7	Up	NA	84.59217478	NA	NA	50.34840675	NA
ZK6.11	Up	13.51671082	9.203800281	11.32765282	25.07211484	9.583548732	55.16639718
F46E10.10	Up	2.391908468	6.045383605	46.44541861	57.899234	NA	20.34578
ZK616.2	Up	17.51832838	17.17780362	22.29255947	67.36631034	NA	52.65035501
Y18H1A.10	Up	81.91983788	18.19256687	39.84178234	72.97638976	16.17469482	40.42372432
Y111B2A.16	Up	37.02852927	6.647337216	38.24872267	88.05696683	2.272715857	61.53891302
ZC395.2	Down	12.27510972	4.067075109	11.80024598	27.29902784	27.11298996	1.214579041
T03F6.3	Down	24.89403644	6.53173818	10.74653438	46.04412991	96.46401222	3.516531323
ZK1025.6	Down	10.34065105	10.03376504	0	17.13876514	NA	0
C04H5.7	Down	0	0	NA	38.25815451	81.03117452	3.192287157
ZK858.3	Down	18.8698461	0	1.349658902	29.40528689	20.87408601	0
C49F8.1	Down	8.543036211	24.10797669	28.30501131	5.931320426	NA	21.81795784
F52D2.4	Down	20.14436489	21.4466519	14.31250596	29.77507937	43.52671082	0
W03C9.7	Down	40.57386939	30.03697954	13.63895315	51.99567694	97.97991817	35.84787235
F45E4.9	Down	16.01221605	6.14503347	39.55826826	28.2241075	27.0523934	45.41615128
B0238.12	Down	33.54217758	33.76595572	17.3404383	20.16518934	NA	17.56392455
AH6.5	Down	NA	NA	NA	NA	NA	75.92348
H38K22.5a	Down	19.36600844	21.35058347	44.15261588	14.00147362	34.0079471	55.39746446

Table 2. The most significant differentially-expressed genes overlapping between *isp-1*, *clk-1* and *cyc-1(RNAi)* mutants. The set of overlapping genes was determined using the "*p-q*" algorithm (see Methods), using the combined set of L4 and adult microarray data as input. Significance values shown were calculated using the SAM software package, also run on the combined set of L4 and adult data. The top 30 genes listed were tested for suppression of lifespan extension in *isp-1* mutants. Also shown are the SAM significance values for these genes using the adult-only set of chips.

3.3.7 - *cdr-2* RNAi suppresses the increased longevity of *isp-1* mutants

`Since *isp-1*, *clk-1* and *cyc-1(RNAi)* mutants are all long-lived, gene expression patterns that are shared between all three might be particularly likely to contribute to lifespan extension. Double RNAi experiments in *C. elegans* can be difficult to interpret, so we did not attempt RNAi knockdowns in *cyc-1(RNAi)* animals. However, we did attempt to knock down the top thirty statistically-significant shared genes, individually, in an *isp-1* background (of these, 21 were not present in the *clk-1* set we described above). We obtained data for 30 of these genes. Of these, only one RNAi clone, *cdr-2,* consistently made our significance cut-off [$p < 0.05$ and a 10% decrease in *isp-1* mutant longevity (which corresponds to a 50% decrease in the lifespan extension produced by the *isp-1* mutation)]. *cdr-2* RNAi reduced the lifespan extension produced by the *isp-1* mutation from 43% (control RNAi) to 26%, while not affecting wild-type longevity (Fig. 10).

cdr-2 encodes a member of the glutathione S-transferase family. These enzymes catalyze the conjugation of reduced glutathione to electrophilic centers on different substrates. This activity contributes to detoxification of both endogenous toxins and xenobiotics, suggesting that the increased longevity of *isp-1(-)* mutants is at least partially dependent on a cellular detoxification response. We note that in one of our three trials, *cdr-2* RNAi

significantly shortened the lifespan extension produced by *clk-1*-mutation (from 21% to 14% longer than wild type). This finding suggests that *cdr-2* may be involved more generally for lifespan extension in mitochondrial mutants.

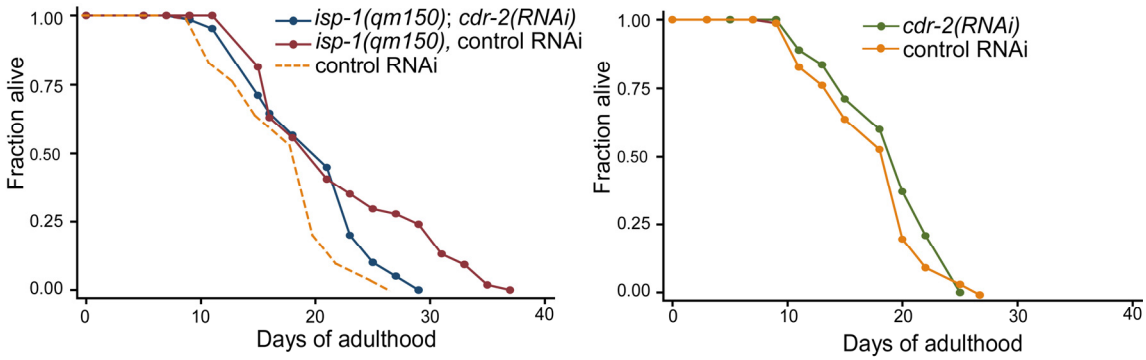


Figure 10 - *cdr-2* RNAi suppresses the increased longevity of *isp-1* mutants. *cdr-2* RNAi suppressed the longevity increase produced by *isp-1* mutation from 43% to 26% ($p < 0.05$) and did not significantly affect wild-type longevity. WT subjected to control RNAi: N = 90, m = 18.1 days; WT subjected to *cdr-2* RNAi: N = 87, m = 19.1 days; *isp-1(-)* subjected to control RNAi: N = 89, m = 22.4 days; *isp-1 (-)* subjected to *cdr-2* RNAi: N = 89, m = 20.1 days. In a second trial, *cdr-2* RNAi decreased the lifespan extension produced by *isp-1* mutation from 24% to 11% ($p < 0.05$). Lifespans were determined at 20°C.

3.3.8 – *fstr-1/2*'s regulatory function is specific to *clk-1* mutants

Given the remarkable reversal of the *clk-1* mutant phenotype by *fstr-1/2* RNAi, we were interested in examining its function in respiration-defective mutants. Unexpectedly, we found that *fstr-1* was significantly up-regulated in *isp-1* mutants but not in *cyc-1(RNAi)* animals. Using RNAi, we asked whether *fstr-1/2* might influence the behavioral phenotypes of *isp-1(qm150)* mutants. We restricted our analysis to lifespan and time to adulthood because *isp-1* mutants did not move often enough to provide consistent

behavioral rates. We found that the development rates of *isp-1* mutants were severely decreased in the presence of *fstr-1/2* RNAi, leading to developmental arrest of many animals. We found that *fstr-1/2* RNAi had no effect on the lifespan of *isp-1* mutants that reached adulthood (Fig. 11). Thus the effect of *fstr-1/2* RNAi on *isp-1* mutants was more similar to the effect of *fstr-1/2* RNAi on wild type than to its effect on *clk-1* mutants.

We wanted to know whether *fstr-1/2* was necessary for gene expression changes in an *isp-1(-)* background, but because *isp-1* mutants subjected to *fstr-1/2* RNAi grew very slowly and asynchronously, we could not use qRT-PCR. Instead, we assayed gene expression *in vivo* by introducing the *isp-1* mutation into a strain expressing GFP under the control of the *gpd-2* promoter, which drives expression of a glycolysis gene that is up-regulated by mitochondrial mutations (Fig. 12). We found that *fstr-1/2* RNAi prevented the up-regulation of this reporter in a *clk-1* background but not in an *isp-1* background. Together, these data suggest that the Isp-1(-) and Clk-1(-) behavioral and longevity phenotypes are established by distinct mechanisms.

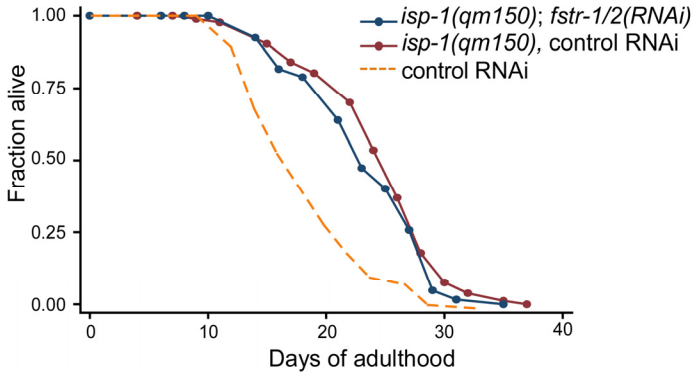


Figure 11 - *fstr-1/2* RNAi did not shorten the lifespan of *isp-1* mutants. *isp-1(-)* subjected to control RNAi: N = 108, m = 24.6 days; *isp-1 (-)* subjected to *fstr-1/2* RNAi: N = 104, m = 23.6 days. These experiments were repeated twice more with similar results (Sup. Table 4).

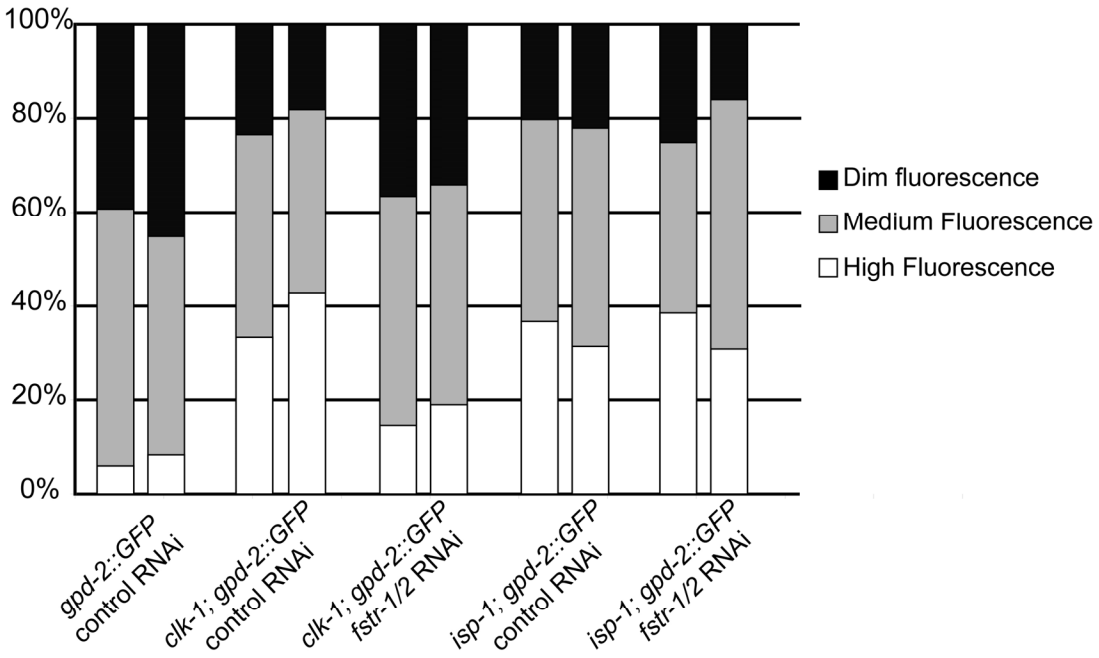


Fig. 12. *fstr-1/2* RNAi does not affect *gpd-2* expression in an *isp-1* mutant. Worms harboring a P*gpd-2::GFP* promoter fusion were ranked as: Dim Fluorescence, Medium Fluorescence and High Fluorescence based on visual inspection. Each bar represents a different population (N = 80 worms). Two separate observations were made for each condition.

3.4 - Discussion

How inhibiting something as fundamental as mitochondrial function can extend lifespan is a profoundly interesting question. *C. elegans* respiration mutants appear to live in “slow motion”, as the rates of a wide variety of processes, including rates of growth to adulthood, aging and behavior, are all reduced. This phenotype is shared by *clk-1* ubiquinone-biosynthetic mutants, which, paradoxically, show only a very mild and temporary decrease in respiration, and have normal ATP levels (Braeckman,

Houthoofd et al. 1999). This spectrum of behavioral and longevity phenotypes is not seen in the many *C. elegans* mutants whose longevity requires the transcription factor DAF-16, or in calorically restricted animals, so these mitochondrial mutants appear to comprise a distinct class of longevity mutants. Reducing *clk-1* activity extends the lifespan of mice, suggesting that a better understanding of these mutants could potentially have implications for human health and longevity.

In this study, we used microarray analysis and RNAi to investigate the mechanisms by which these various mitochondrial mutations acquire their distinctive behavioral and longevity phenotypes.

3.4.1 - A C. elegans retrograde response

Gene expression profiling of these mutants was quite revealing, because each of their expression profiles exhibited striking similarity to the yeast retrograde response. In yeast, the retrograde response appears to remodel the cell's metabolism. Without respiration, the Krebs cycle cannot be completed, as succinate cannot be oxidized to fumarate (Fig. 13). This prevents the formation of oxaloacetate (OAA), which in turn decreases the availability alpha-ketoglutarate, which is the precursor of glutamate, an essential metabolite in amino acid metabolism. In order to recycle the precursors of glutamate, respiration-deficient cells must activate alternative (anaplerotic) pathways that supply the mitochondria with OAA and acetyl-CoA (Butow and Avadhani 2004). Activation of anaplerotic pathways was observed in respiration-defective yeast (Epstein, Waddle et al. 2001; Traven, Wong et al. 2001) and human cells (Miceli and Jazwinski 2005).

Our microarrays reveal activation of several metabolic enzymes that have roles in anaplerotic reactions, such as the glyoxylate cycle and fatty acid oxidation. The glyoxylate cycle occurs mainly in the peroxisomes and bypasses the succinate-to-fumarate step of the Krebs cycle through the

formation of glyoxylate, eventually leading to the formation of succinate, which can be fed back into the Krebs cycle (Fig. 13). We observed increased expression of the gene encoding the major *C. elegans* glyoxylate-cycle enzyme, GEI-7, in the three mitochondrial mutants we examined. We did not observe an RNAi phenotype for this clone in the *clk-1* mutant; however, because a *gei-7* mutant was available, we examined *cyc-1(RNAi); gei-7* animals and found a large suppression of the *cyc-1(RNAi)* longevity phenotype, decreasing lifespan extension from 80% to 15% with little effect on wild type (Sup. Fig. 3). Malate dehydrogrenases catalyze synthesis of OAA from malate, which is also an important step in recycling Krebs cycle intermediates. F46E10.10 encodes a malate dehydrogenase and is significantly up-regulated in all three long-lived mitochondrial mutants we examined. Fatty-acid oxidation provides acetyl-CoA, which feeds into the Krebs cycle by reacting with OAA to form citrate. This pathway is activated in long-lived yeast lacking mitochondrial DNA (Butow and Avadhani 2004). We also detected increased expression of several genes that are involved in fatty acid oxidation. *clk-1(-)* animals exhibited increased expression of *acs-2* (acetyl-CoA synthetase) and *fat-6* (fatty acid desaturase); and *isp-1* mutants increased expression of T02G5.4 (acetyl-CoA thiolase) and T05G5.6 (Enoyl-CoA hydratase). The expression profiles of *cyc-1(RNAi)* animals, however, contained fewer significant genes involved in fatty acid oxidation, suggesting there may be some differences in metabolic adjustments between different mitochondrial mutants.

In all three long-lived mitochondrial mutants, we observed a significant increase in expression of genes involved in glycolysis. This was expected, since glycolysis becomes a more important source of ATP when oxidative phosphorylation is inhibited.

In addition to these metabolic shifts, we also observed increased expression of a significant number of stress response genes in all three mitochondrial mutants, ranging from genes increasing xenobiotic drug-

resistance to protein chaperones. This is consistent with previous *in vitro* observations that impairment of electron flow during oxidative phosphorylation is actually likely to generate more ROS (Lenaz 2001), and suggests these animals may be responding to this additional cellular insult.

Finally, in all three strains, genes involved in the oxidative phosphorylation process itself were up-regulated, and we observed increased levels of mitochondrial DNA in *clk-1* and *isp-1* mutants. Thus, apparently *C. elegans* mitochondrial mutants, like yeast (Traven, Wong et al. 2001), attempt to compensate for reduced mitochondrial efficiency. Together these findings show that the retrograde response triggered in *C. elegans* is very similar to the one present in long-lived respiration-defective yeast and suggest the presence of a conserved pathway regulating longevity in response to mitochondrial dysfunction.

While this manuscript was in preparation, Falk *et al.* reported the gene expression pattern of a mixture of long-lived and short-lived respiration-defective mutants compared to wild type (Falk, Zhang et al. 2008). In the future, it will be interesting to learn whether the expression patterns of short-lived mitochondrial mutants differ from those of the long-lived mutants. The lifespan of one such short-lived mutant, *mev-1(kn1)* is increased when respiration is lowered further using respiratory-chain RNAi (Fig. 14), arguing that their short lifespans are not due simply to insufficient respiratory-chain activity.

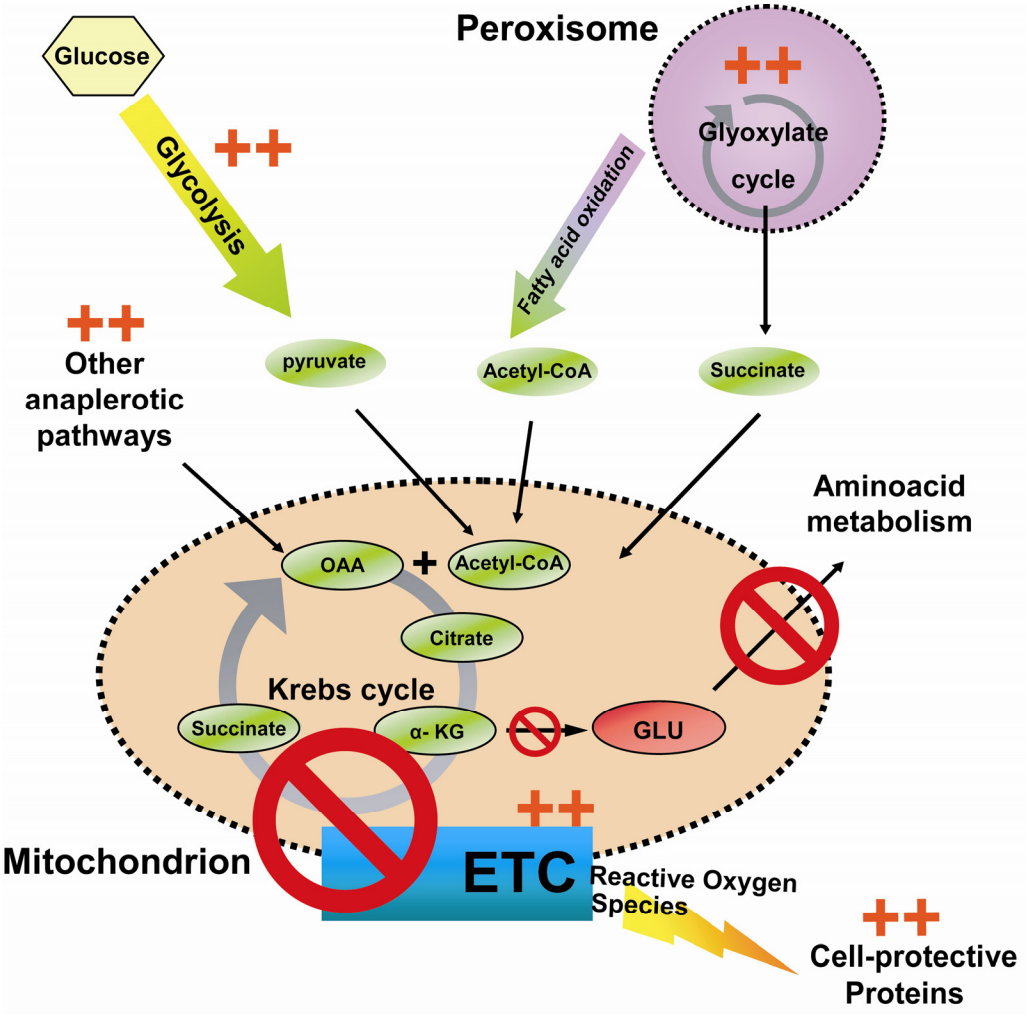


Figure 13 - *C. elegans* retrograde response. The picture diagrams metabolic changes thought to occur during the yeast retrograde response (Butow and Avadhani 2004). "++" indicates that these same changes are observed in the *C. elegans* retrograde response; Impairment of electron flow during oxidative phosphorylation is predicted to have two effects. The first is an increase in ROS, due to increased likelihood of electrons transferring to free oxygen (Lenaz 2001). Consistent with increased ROS stress (which we did not assay directly), we observed an increase in cell-protective genes. Secondly, the Krebs cycle is disrupted because the enzyme succinate dehydrogenase is an integral part of both the electron transport chain and of the Krebs cycle. When succinate

dehydrogenase oxidizes succinate into fumarate it feeds electrons into the ETC, and when this flow is blocked, the enzyme's activity is inhibited. The Krebs cycle is necessary for synthesis of glutamate, which in turn is required for amino acid metabolism. Impairment of the electron transport chain leads to decreased glutamate production. Possibly, in order to counteract this decrease in glutamate production, the cell induces anaplerotic pathways that feed intermediates into the Krebs cycle, thus allowing production of glutamate. Under conditions of impaired respiration, glycolytic gene expression increases, consistent with glycolysis' becoming a major source of ATP. Consistent with a compensatory response, expression of genes involved in oxidative phosphorylation is up-regulated. OAA, Oxaloacetate; GLU, glutamate; ETC, electron transport chain; α-KG, α-ketoglutarate.

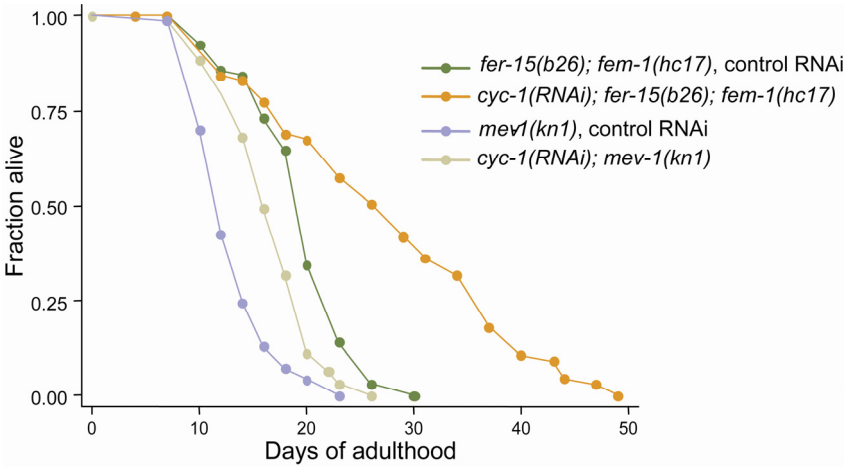


Figure 14 - The short lifespan of *mev-1* mutants is increased by respiratory-chain RNAi. WT subjected to *cyc-1* RNAi: N = 81, m = 27.6 days; WT subjected to control RNAi: N = 81, m = 19.7 days; *mev-1(kn1)* mutants subjected to *cyc-1* RNAi: N = 80, m = 16.7 days; *mev-1(kn1)* mutants subjected to control RNAi: N = 78, m = 13.2 days. This lifespan analysis was performed twice, $p < 0.001$ both times.

3.4.2 - Long-term reductions in respiration are not necessary to maintain expression of the retrograde response

It was interesting to find that *clk-1* mutations trigger a conserved retrograde response even though they only have a mild effect on respiration. There are at least two possible interpretations for this finding. The first is that the retrograde response need not be triggered by reduced respiration itself, but instead can be triggered by signals that are generally associated with reduced respiration, such as fluctuations in ubiquinone levels. Such fluctuations could have acquired the ability to induce the retrograde response during evolution because they allowed the animal to conserve energy in the face of a perceived energy shortage. Alternatively, perhaps the *clk-1* mutation does inhibit respiration initially, but the physiological changes elicited by the retrograde response (such as increased mitochondrial biosynthesis), restore the steady-state level of respiration to normal.

3.4.3 - The retrograde response is probably required for the longevity of C. elegans mitochondrial mutants.

In yeast, the retrograde response is induced via helix-loop-helix transcription factors that do not appear to be present in *C. elegans.* When the genes encoding these transcription factors are deleted in yeast, inhibiting respiration does not induce the retrograde response, and lifespan is not extended (Kirchman, Kim et al. 1999). Thus, in yeast, the retrograde response likely increases lifespan. Our data suggest that this is the case in this multicellular animal as well. First, inhibiting the activity of at least some of the genes up-regulated in mitochondrial mutants was sufficient to shorten their lifespan without obviously affecting the lifespan of wild type. In particular, the glutathione S-transferase gene *cdr-2* was up-regulated in all of the long lived mutants and it contributed to lifespan extension

consistently in *isp-1* respiration-defective mutants and, at least in some trials, in *clk-1* mutants as well. This finding suggests that the prominent cell-protective gene expression response that we observe contributes to longevity. In addition, the metabolic shifts we observed are also likely to influence lifespan, as the longevity of *cyc-1(RNAi)* animals required the glyoxylate-cycle gene *gei-7* (Fig. 15).

Our failure to observe effects on lifespan with most of the RNAi clones we tested does not necessarily mean that they do not influence lifespan (though this may be the case). It seems likely that many of these genes would act cumulatively to influence lifespan. The lifespan extension of *clk-1* and *isp-1* mutants was only ~20% in most experiments, so only perturbations that are fairly strong would be visible in our assays.

The second argument for the importance of the *C. elegans* retrograde response in the longevity of mitochondrial mutants comes from our studies of *fstr-1/2*. *fstr-1* was up-regulated in *clk-1* mutants, and this gene, and/or its constitutively-expressed homolog *fstr-2,* is required, in turn, for a robust retrograde response. Knocking down *fstr-1/2* activity with RNAi did not suppress the primary ubiquinone defect. However, none of the five up-regulated genes we tested was up-regulated in the presence of *fstr-1/2* RNAi. These genes included metabolic as well as cell-protective genes, arguing that *fstr-1/2* is a major regulator of the retrograde response in *clk-1* mutants. This restoration of a normal transcriptional profile correlated with a suppression of the behavioral, growth and longevity phenotypes of *clk-1* mutants. Together all of these findings support the hypothesis that the conserved mitochondrial retrograde response extends lifespan in metazoans as well as in yeast. We note, however, that *fstr-1/2* RNAi had stronger effects on the induction of the retrograde-response genes we tested than it had on the *clk-1* behavioral phenotypes. This suggests either that part of retrograde response is expressed independently of *fstr-1/2*, or

that mechanisms that do not involve transcription also influence the *clk-1* phenotype.

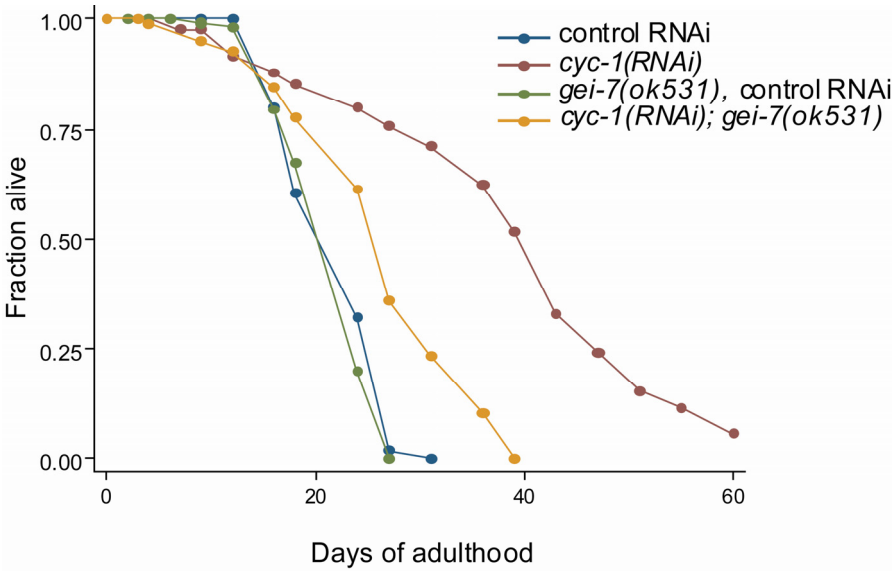


Figure 15 - The glyoxylate cycle gene *gei-7* is partially necessary for *cyc-1* RNAi to increase longevity. WT subjected to *cyc-1* RNAi: N = 109, m = 40.1 days; WT subjected to control RNAi: N = 113, m = 22.3 days; *gei-7(ok531)* mutant subjected to *cyc-1* RNAi: N = 118, m = 26.6 days; *gei-7(ok531)* mutant subjected to control RNAi: N = 119, m = 22.1 days. This lifespan analysis was performed twice, $p < 0.001$ both times.

3.4.4 - The molecular function of FSTR-1/2

How does FSTR-1/2 regulate gene expression? Little is known about the molecular function of FSTR-1/2. FSTR-1/2 contains 21 ET modules, which are domains of unknown function containing 8-10 conserved cysteines predicted to form 4-5 disulphide bridges. Sequence alignment studies using the BLAST algorithm showed weak similarities to a secreted yeast protein (*AGA1*) and a predicted mouse membrane protein (Zonadhesin); however, we found no clear secretion signal or

transmembrane domain. We also looked for structural homologues of *fstr-1/2* using the software package PHYRE (Bennett-Lovsey, Herbert et al. 2008) and found highly significant predicted structural similarities to portions of ErbB1/2/3/4. ErbB proteins belong to a highly conserved family of receptor tyrosine kinases that play many roles in cell biology and disease. Members of the ErbB family usually contain an extracellular region (~ 620 amino acids) that recognizes and binds ligands, a single membrane spanning region and an intracellular tyrosine kinase domain. However, we did not find a predicted tyrosine kinase domain or a secretion signal in *fstr-1/2*'s sequence, which makes a potential connection to the ErbB family unclear. Finally, FSTR-1 (originally called GFI-1, for GEX-interacting protein) was identified in a two-hybrid screen as a potential binding partner of UNC-68, a muscle-specific *C. elegans* ryanodine receptor. Our attempts to find phenotypic similarities or functional interactions between these two proteins were unsuccessful (data not shown), so their potential relationship remains unclear.

Our findings suggest that FSTR-1 expression in the intestine and/or nervous system may influence the longevity of *clk-1* mutants. Because the whole animal is affected by the *clk-1* mutation, it is likely that FSTR-1 either is, or controls, a secreted signal that regulates the physiology of the entire animal. Alternatively, perhaps FSTR-2 acts in other tissues to influence lifespan. An attractive model would be that changes in ubiquinone synthesis activate expression of *fstr-1* in neurons and intestine. FSTR-1 (and possibly also FSTR-2) then activate the broader retrograde response, which in turn reduces growth and behavioral rates and extends the lifespan of the animal (Fig. 16).

3.4.5 - Different paths to a similar phenotype

Long-lived *C. elegans* mitochondrial mutants share many phenotypes; however, there are also some significant differences between them (in respiration rates, ATP levels and body size). These differences have prompted the question of how similar the *C. elegans* mitochondrial mutants really are to each other (Hekimi and Guarente 2003). Our microarray observations suggest that the overall nature of the response is similar, though the specific genes and the extent to which they are activated varies between mitochondrial mutants. Perhaps these differences are phenotypically significant; for example, *clk-1* mutants may have normal respiration rates because they can compensate more fully than the other mutants to a primary respiratory-chain defect. On the other hand, there is a clear difference in the regulation of the *clk-1* and *isp-1* mutant phenotypes, since *fstr-1/2* is necessary for the *clk-1* mutant phenotypes but not for the *isp-1* mutant phenotypes (Fig. 16). It will be interesting to learn how the *isp-1* and cyc-1 retrograde responses are regulated, and at what point these pathways converge to control the same downstream genes.

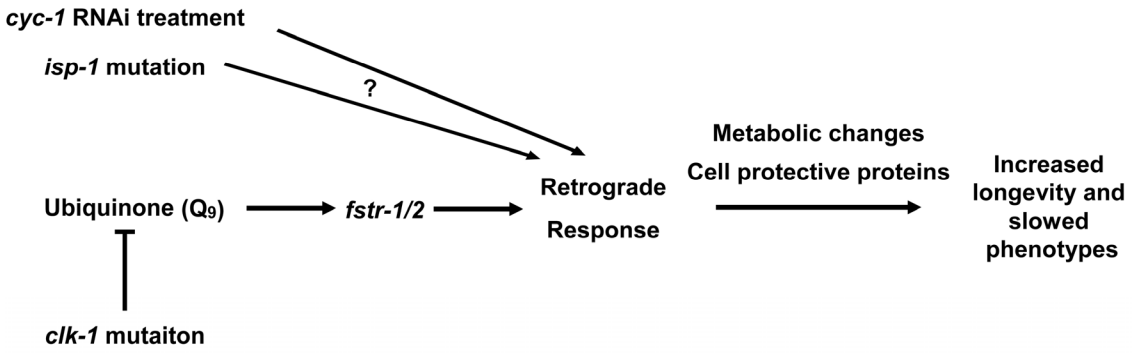


Fig. 16 - *C. elegans* mitochondrial mutants activate similar retrograde responses in different ways. In this model *clk-1* mutation decreases ubiquinone levels, which increases *fstr-1* expression (possibly with the assistance of *fstr-2,* which is expressed constitutively). *fstr-1/2* are then necessary for expression of

other genes that are part of the retrograde response, which in turn are likely to be responsible for the longevity and slowed phenotypes. *clk-1*, *isp-1* and *cyc-1(RNAi)* mutants show similar retrograde responses, however, *fstr-1/2* is necessary for the retrograde response of *clk-1* but not *isp-1* mutants.

3.5 - Methods

Strains

The strains used in this study were: N2-Bristol (WT), *fer-15(b26)*; *fem-1(hc17)*, *clk-1(qm30)* (Wong, Boutis et al. 1995), *isp-1(qm150)*(Feng, Bussiere et al. 2001), *gei-7* (ok531), *mev-1(kn1)*(Ishii, Takahashi et al. 1990), muEx491[p*fstr-1*::mCherry + p*odr-1*::CFP], *clk-1(qm30)* ; muEx491, sEx11128[pgpd-2::GFP], *isp-1(qm150)*; sEx11128[p*gpd-2*::GFP]. All strains used except for *mev-1(kn1)* were outcrossed to the laboratory's N2 4 times.

Microarray hybridizations

We constructed microarrays using single-strand DNA oligos representing 20,374 unique *C. elegans* genes. These were purchased from Illumina® (DeRisi, Iyer et al. 1997). Populations were starvation-synchronized as L1s overnight and collected at two different times: as L4s staged based on vulval morphology and as pre-fertile adults soon after the L4-to-young-adult molt, to guarantee maximum synchronicity between animals that grew to adulthood at different rates. Hybridizations were performed using standard techniques described in (Murphy et al. 2003). Total RNA was purified using TriZol™ reagent, mRNA was purified using Oligotex (Qiagen) and cDNA was labeled using Cy-dyes prior to hybridization. All chips described are direct comparisons between N2 and either *clk-1(qm30)*, *isp-1(qm150)* or *cyc-1(RNAi)* in L4-staged or adult populations. We performed four independent biological repeats for each condition with the exception of *clk-1(-)* and *isp-1(-)* L4-staged populations, where we only collected data for 2 biological repeats. Dye-swaps and

technical repeats were averaged and analyzed as one biological repeat. Scanning was done using a GenePix 4000B scanner, and initial spot quality check was done using Genepix 6.0 software. During the analysis we used 2 different sets of chips for each mutant: the “combined set” includes a combination of all L4 and adult chips, and the “adult-only set” only includes chips from populations collected as adults.

Significance analysis

The microarray data were analyzed twice over the several-year period spanned by these studies. The initial analysis, used to generate candidate genes that may be functional in the extended longevity of mitochondrial mutants, was performed on the combined set (L4 and adults) of microarrays. In this analysis, standard ratio-based normalization and default program settings for flagging missing or “bad” spots were used in the Acuity 4.0 software package. Gene significance was calculated using the SAM software package. All of the lifespan analysis described herein was based on genes at the top of the list when the data were analyzed in this way. The second analysis, used to generate genes for use in the comparative GO analysis, was performed on the adult-only set of arrays. These data were renormalized using lowess as well as ratio based normalizations. On the assumption that genes within the same operon should in general have similar expression patterns, flagging parameters were adjusted to those that maximized the expression correlation of genes within the same operon on a “training” subset of the arrays. Genes not present in at least 3 of the arrays were not considered. These data were analyzed using the SAM software package and genes were considered significant below a FDR (false discovery rate) of 0.1. Data analyzed in this way were used for the GO analysis.

Gene set overlap analysis

An algorithm, which we named the "*p*-*q* algorithm", was designed and implemented in the Python programming language to determine the set of genes that are differentially regulated in all three combined (L4 and adult) microarray data sets. The algorithm takes as input a set of microarray hybridization data and estimates the *q-value* for each gene using the method described in (Storey 2002) and *p-values* estimated using Student's t-test. It then iterates through each *q-value* and calculates the probability of seeing the observed number of genes that would be significant in all three data sets, should that *q-value* be used as the threshold for significance. The algorithm reports the set of genes that overlap between the three data sets at the *q-value* cut-off that achieved maximum overlap significance, as well as the probability of seeing such a degree of overlap by random chance. Probabilities are calculated using the hypergeometric distribution when possible, or the Poisson approximation when necessary.

GO analysis

GO categories were found using the BINGO software starting from a list of differentially expressed genes obtained from running SAM on the set of adult-only microarrays, with a significance cut-off of FDR <= 0.1 for each *C. elegans* mutant. Yeast GO categories were obtained by analyzing a dataset pooling differentially genes from two different publications (Epstein, Waddle et al. 2001; Traven, Wong et al. 2001).

RNAi

Bacterial feeding RNAi experiments were performed as described previously (Murphy et al. 2003). Clones were picked from Julie Ahringer RNAi library and were verified by sequencing.

Survival measurements

Lifespan analysis was conducted as previously described (Dillin, Hsu et al. 2002). All assays were done at 25°C unless otherwise stated. The Stata 8.0 software package (Stata Corporation) was used for statistical analysis and to calculate means and percentiles. In all cases p-values were calculated using the logrank (Mantel-Cox) method.

Mitochondrial DNA quantification

Mitochondrial DNA was quantified using Real Time-qPCR. We used two primer sets for mitochondria DNA graciously provided by Dana Miller:

Mito1: Forward:GTTTATGCTGCTGTAGCGTG,

Reverse:CTGTTAAAGCAAGTGGACGAG;

Mito2: Forward:CTAGGTTATATTGCCACGGTG,

Reverse:CAATAAACATCTCT-GCATCACC.

The results were normalized to genomic DNA using a primer pairs specific for *ama-1* and *nhr-23*:

ama-1: Forward: TGGAACTCTGGAGTCACACC,

Reverse: CATCCTCCTTCATTGAACGG;

nhr-23 : Forward:CAGAAACACTGAAGAACGCG,

Reverse:CGATCTGCAGTGAATAGCTC.

Animals were grown and collected as described above for microarray studies and lysed in a standard buffer containing proteinase K for 1 hour at 65°C. qPCR was performed using SYBR® GREEN PCR Master Mix (Applied Biosystems). Each comparison pools 5 biological repeats.

Rates of growth and behavior

Time to adulthood was measured as time (± 2 hours) at which 95% of animals reached adulthood. Measurements shown represent pooled data from five independent experiments, error bars represent SEM. Pumping rate was measured as the average number of pharyngeal pumps per minute (n = 10) over three independent trials. Thrashing rate was measured

as the average number of body thrashes in M9 buffer in one minute (n = 10) in three independent trials. All measurements were conducted on three day old adult animals.

Quantitative RT-PCR

Real-time RT-PCR was carried out using the 7300 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). Primers and probes were designed specifically for each gene using Primer3 software.

Construction of the Pfstr-1::mCherry promoter fusion

To generate P*fstr-1*::mCherry-expressing animals, a P*fstr-1*::mCherry construct was made using the Invitrogen Gateway® Cloning technology, the promoter was amplified from genomic DNA using a primer set obtained from Mark Vidal's online promoterome database (Forward: ggggacaactttgtataga aaagttgaggccagctttagataat; Reverse: ggggactgctttttttgtacaaacttgtcatctgaaatt tgaatgtgttagt). The construct obtained was sequenced and injected as described (Mello and Fire, 1995) at 10 ng/µl into N2 animals to generate a transgenic line (indicated by muEx491 designation). The coinjection marker P*odr-1*::GFP was injected at 50 ng/µl.

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3.7 - Author Contributions

David Cristina carried out most of the experiments and the initial microarray analysis. Michael Cary, a Biomedical Informatics Ph.D. student, developed and applied the technique of optimizing normalization parameters based upon expression correlation of genes in the same operon, and carried out the GO analysis. Adam Lunceford and Catherine Clark measured ubiquinone species in *clk-1(-); fstr-1/2(RNAi)* animals. Cynthia Kenyon provided guidance. DC and CK wrote the paper.

Chapter IV
Final Considerations on Mitochondria and Aging

Aging research has made significant advances in the last 15 years with the discovery of many genes that regulate longevity, somewhat surprisingly so, considering what evolutionary theories had anticipated. Roughly, these genes can be grouped into three distinct pathways: Insulin/IGF-1 signaling and other DAF-16/FOXO-dependent pathways, dietary restriction and mitochondrial inhibition. The most recently identified and least understood of the three is the mitochondrial pathway.

Prior to the work presented here, little was known about how mitochondrial mutations affected longevity and no genetic regulators of this pathway had been identified. In addition, although there was some evidence supporting evolutionary conservation of the relationship between mitochondria and increased longevity, no mechanism had been identified that would explain said connection. The work presented in this thesis identifies retrograde regulation as a likely candidate for the conserved mechanism linking mitochondria to increased longevity. Long-lived yeast cells with impaired mitochondrial function have a well-documented retrograde response that is necessary for their increased longevity. The data presented here suggests that in *C.elegans,* the mitochondrial mutants' increased longevity is a direct consequence of the activation of a retrograde response very similar to the one present in yeast. Several lines of evidence support this: Firstly, the expression profiles of these long-lived *C. elegans* mitochondrial mutants are highly similar to the ones observed in long-lived yeast cells. Secondly, knocking-down some of the genes up-regulated by this mitochondrial retrograde response (*aqp-1*, *fstr-1/2* and *cdr-2*) in

mitochondrial mutants leads to a significant decrease in longevity, thus arguing that these genes are necessary and, very likely, partially responsible for the long-life phenotype of these animals. Thirdly, *fstr1/2*, a suppressor of *clk-1* phenotypes, is necessary for at least several genes in the worm's retrograde response to be induced, providing further evidence that the retrograde response has a likely role in longevity. These findings suggest an important and evolutionarily conserved role for retrograde regulation in longevity. Since retrograde regulation has been described in mammalian cells, it would be very interesting to test whether the long-lived mouse mitochondrial mutants *Mclk-1*+/- have similar expression profiles to the ones found in yeast and worm long-lived mitochondrial mutants. Also, comparing expression profiles of these long-lived mitochondrial mutants to the expression profiles of other long-lived mutants (IIS, DR) is likely to shed light on important common regulators of longevity.

As for how mitochondrial impairment can actually lead to increased longevity, the gene expression profiling studies together with RNAi knock down data suggest a role for stress response and metabolic rearrangements (see section 3.4.3).

cdr-2 is an important stress response protein and is necessary for *isp-1* mutants' increased longevity, also *gei-7*, essential for the glyoxylate cycle, is necessary for *cyc-1(RNAi)* treated animals to live long. It is tempting to speculate that since the mitochondria are the main source of ROS, which are a likely cause of aging, localized increased expression in stress response genes may ameliorate this oxidative damage. It is also possible that the mitochondria act as a cellular stress sensor. In this case, by impairing mitochondrial function we are "tricking" cells into activating a robust stress response. This response, in turn, overshoots the added damage of the mutations and leads to an increase in longevity. It is interesting that several xenobiotic response genes are induced (see section 3.3.1 and 3.3.6) in these mitochondrial mutants. It would be important to

determine what role these genes have in lifespan and whether these long-lived mitochondrial mutants show increased resistance to xenobiotics. Consistent with this, a role for xenobiotic resistance in longevity has already been proposed regarding the insulin/IGF-1 pathway (McElwee, Schuster et al. 2004).

An interesting hypothesis on how metabolism could be influencing longevity also takes into account cellular oxidative damage. Since the ETC and cellular respiration are important sources of ROS, then, activation of alternative metabolic pathways could lead to decreased oxidative damage. It is possible that the alternative metabolic pathways that are active in these long-lived mitochondrial mutants generate less ROS than mitochondrial respiration, and in that way, extend the animals' longevity. It is important to note that these alternative pathways also generate significantly less energy than mitochondrial respiration, and for that reason are less likely to be active under normal circumstances. From this, we can speculate that animals are optimized for energy production, not longevity. As a follow-up to this study, it would be interesting to use standard biochemical assays to determine with certainty what metabolic pathways are active in these mutants. From there, it would be possible to supplement the media with certain intermediate metabolites to try to understand whether the hypothesis proposed in this thesis is correct, namely, whether glutamate is a central metabolite in the retrograde response (See section 3.4.1).

Importantly, this work identified *fstr-1/2*, which is necessary for the increased longevity of *clk-1* mutants. This is the first player in a potential pathway connecting mitochondria and longevity. It would be interesting to see if *fstr-1/2* also has a role in other longevity pathways such as insulin/IGF-1 or dietary restriction. Such experiments could establish it as a more general mechanism regulating longevity in *C. elegans.*

The "slowness" of these long-lived mitochondrial mutants has been suggested to be a passive consequence of decreased energy availability

caused by lowered mitochondrial function. The increase in longevity observed would have a similar explanation, where decrease in metabolic output would also passively lead to less ROS production. The fact that knockdown of a regulatory molecule, *fstr-1/2*, increases movement rates irrespective of energy availability suggests that these mitochondrial mutants actively "slow down" their behavioral rates. It is possible that this slowing down is an attempt to adjust the organisms' energy expenditure to its energy production ability. Most importantly, however, this suggests the existence of a physiological mechanism responsible for adjusting rates-of-living to energy expenditure.

Based on this model, it is interesting to note that the important stage for the decision affecting longevity and behavioral rates is late in development (see chapter II), if mitochondrial impairment occurs only in adulthood no longevity benefit is observed. During development, animals make certain developmental choices depending on the environmental conditions such as temperature and energy availability. It is tempting to speculate that this regulation of rates-of-living in response to energy availability is one of these decisions, which also has implications on aging and longevity. Significantly, these findings also argue that events occurring early in development can influence longevity during the animal's whole life. An interesting question with potential implications for therapeutic design is whether this lifespan extending retrograde pathway can also be activated in adults animals.

A somewhat surprising observation was that *fstr-1/2*'s effect on longevity is specific to *clk-1* mutants. As shown in section 3.3.8 *fstr-1/2* is not needed for respiration deficient mitochondrial mutants to live long. This further reinforces the notion that although these long-lived *C.elegans* mitochondrial mutants share several similarities there are some significant differences (see section 1.4.3). Conversely, they do share similar transcription profiles. Based on this, a likely scenario is that they activate similar responses modulating longevity, but do so through different

mechanisms. In the future, it would be interesting to look for molecules regulating the longevity of these respiration deficient mitochondrial mutants.

fstr-1 is expressed in neurons and in the intestine, however its effects are on the whole organism. Bearing this in mind, because the whole animal is affected by the *clk-1* mutation, it is likely that FSTR-1 either is, or controls, a secreted signal that regulates the physiology of the entire animal. Alternatively, perhaps FSTR-2 acts in other tissues to influence lifespan. An attractive model (see section 3.4.4) proposes that decreases in ubiquinone levels signal an increase in *fstr-1/2*, which contributes to the activation of a retrograde response. This, in turn, leads to increased longevity. However, the mechanism of action of *fstr-1/2* still requires much elucidation, and this is essential for the understanding of this pathway. Further biochemical characterization of *fstr-1/2* would likely shed light on the mechanisms by which it is helping to promote longevity, and potentially allow the identification of other players in this pathway.

Overall, this work provides very significant insight into a longevity pathway that was almost completely unexplored. It has addressed some of the broader questions regarding mitochondria and longevity in *C. elegans*. In doing so, however, it inevitably generated more questions than answers. These findings represent a solid stepping-stone on the way to understanding the complex relationship between mitochondria and longevity.

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